University of Alberta

The Effects of Introducing Fructose during Suckling on Body Weight in Adulthood

BY

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Abstract

High dietary fructose consumption can result in detrimental health consequences. However, most studies examined immediate metabolic outcomes following a period of high fructose feeding. The purpose of this study is to determine the potential programming effects of early fructose intake on metabolism as it pertains to body weight in adulthood. Two groups of experiments were carried out using the same study design. Rat pups were artificially reared from 12 – 19 days of age. Suckling diets consisted of: 1) LAC (lactose as the sole carbohydrate, 2) FR (1:1 lactose:fructose), 3) GAL (1:1 lactose: galactose), 4) SC (suckle controls). Total carbohydrate content of all diets was equal. Pups were weaned to a purified rat chow until 77 days of age. Results from group #1 demonstrated that fructose-feeding led to increased long chain fatty acid uptake into skeletal muscle, higher circulating insulin and leptin concentrations along with increased body weight in adulthood. Results from group #2 did not show differences in outcome parameters between dietary treatments. In conclusion, the effects of a short period of high fructose intake during suckling on adult body weight are subtle. In future experimental designs, re-introduction of the high fructose feeding regime in the post-weaning period may facilitate the expression of the effects of early fructose ingestion.

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Abbreviation list

AR – artificial rearing

ARC – arcuate nucleus center

AS – artificial sweetener

ATP – adenosine triphosphate

CCK – cholescystokinin

CNS – central nervous system

FFSc – fructose-fed suckle control

(suckle control group that was fed 65% FR at 8 weeks of age)

FR – fructose

G3P – glyceraldehyde-3-phosphate

GAL – galactose

HC – high carbohydrate

HFCS – high fructose corn syrup

LCFA – long chain fatty acid

LL – large litter

LPL – lipoprotein lipase

MIRKO – muscle specific insulin receptor knockout

NIRKO – neuron specific insulin receptor knockout

NL – normal litter

NPY – neuropeptide Y

OGTT – oral glucose tolerance test

PVN – paraventricular nucleus

RMS – rat milk substitute

SC – suckle control

SEM – standard error of estimate

SL – small litter

TG – triglyceride

UCP 1 – uncoupling protein 1

VLDL – very low density lipoprotein

Chapter 1. Literature review

1. Introduction

Within the past two decades, the prevalence of obesity has increased substantially, prompting a closer look at the possible causes of this problem. In the US, approximately 64% of the adult population is overweight and 31% are clinically obese (1). Within Canada, these trends are also present, albeit to a lesser extent with 51% of Canadians classified as overweight and 15% as obese (2). The etiology of obesity is multi-factorial and can include societal and family factors, predisposing genetics, along with factors related to diet and physical activity (3). In terms of diet, excessive fat consumption has been implicated in causing obesity. However, the prevalence of obesity has greatly increased, despite an apparent decrease in fat consumption (4). In the US, between 1976 and 1991, the mean fat intake as a percentage of total calories decreased from 36% to 34% (5). Therefore, the effects of other dietary factors on body weight should be considered. One such factor is dietary fructose (FR) as the rise in consumption of this sugar coincides with the increased rate of obesity within the past two decades (6,7).

Currently, there is building interest in programming effects of early nutrition on later health. It has been proposed that nutritional conditions during critical developmental stages can influence disease onset in maturity. Although the body of evidence is growing, there is a significant gap in knowledge concerning the time frame, duration of feeding and type of diet that could influence susceptibility to adulthood diseases. The purpose of this literature review is to explore the bodies of knowledge that

have accumulated in both programming and FR metabolism in relationship to metabolic consequences. An emphasis will be placed on alterations in body weight.

2. Origins of adulthood disease

According to the Thrifty Phenotype hypothesis, there is an association between the nutrient environment during fetal and early years of life and subsequent risk of chronic diseases in maturity (8). Briefly, these investigators propose that a primary insult, possibly due to maternal malnutrition or under-nutrition during gestation, can change organ structure, physiology and function in the offspring. These alterations can persist into adulthood thereby increasing the likelihood of chronic disease in later life. As animals age, exposure to permissive environments, such as excessive calories and reduced energy expenditure, results in early manifestation of chronic diseases.

The association between nutritional modification during the prenatal period and later risk of chronic diseases has been well-characterized (9,10). Most notably is the illustration between imprinting due to gestational under-nutrition and impairment of glucose homeostasis in later life (11,12,13). For example, individuals who were in utero during the Dutch Famine have significantly poorer glucose tolerance than those who were born a year before the famine (14). However, less work has been done in the postnatal period to examine the effects of dietary manipulation

3. Animal models

The relationship between early postnatal dietary manipulation and weight changes in later life is complex because of the multi-factorial nature of body weight. Adult body

weight represents the cumulative interactions among physiological, genetic, social, behavioral and environmental inputs. In animal models, some of these complexities are controlled under strict standard laboratory conditions, thus researchers are able to focus on dietary manipulations alone on outcome parameters. Also, in the context of examining the role of dietary manipulations on later body weight, ethical considerations prevent researchers from conducting clinical investigations in humans whereby sub-optimal nutritional conditions are part of the study design. This shows the necessity for animal models in order to study these effects.

3.1. Postnatal dietary manipulations

The postnatal period can be defined as "occurring or being after birth" (15). Due to ethical considerations, human data in this area is scarce. Two studies that documented the effects of under-nutrition in premature infants took place in the United Kingdom between 1982 – 1985 (16, 17). Essentially, these investigations compared the effects of common sources of nutritional support in the 1980s - banked breast milk and term formula versus a new nutrient-dense preterm formula. Because of the inconclusive nature of clinical data, investigators were permitted to randomize preterm infants to either the lower nutrient diets or to the more nutritious preterm formula. Data from these studies illustrated that early postnatal accelerated weight gain due to higher calorie intake resulted in increased risk of insulin aberrations (16) and development of obesity and cardiovascular diseases in adolescence (17).

In terms of animal models, researchers have examined two different routes of dietary manipulation during the suckling period: 1) macronutrient modification via the

artificial rearing (AR) technique and 2) total nutrition modification by manipulation of litter size.

3.1.1. Litter manipulation- effects on body weight

Litter manipulation involves the redistribution of pups shortly after birth, to either large or small litters. The premise is based on the positive association between the amount of milk the suckling animal ingests and its growth rate. McCance (18) demonstrated this concept by distributing pups from two separate litters that were born on the same day to suckle on their dams. The ratios of pups to dam were 3:1 in the small litter (SL) and 15:1 in the large litter (LL). He observed that at 3 weeks of age, the pups that suckled in groups of three were two to four times the weight of those suckled in groups of fifteen. Similar results were demonstrated in mice by Aubert et al. (19). Their method was similar to McCance, however, here; there were three suckling groups – four, nine and twenty pups per dam. Litters of nine served as the normal size group (NL), corresponding to natural litter size in Swiss strain mice. At 28 days of age, the mice that suckled in SL were heavier compared to both, the NL and LL pups. This pattern of growth continued until they were 18 weeks old (SL: 53.3 ± 1.5, NL: 46.9 ± 1.2, LL: 40.6 ±0.7 g). Body fatness was also significantly different as a result of over/under nutrition. Mice raised in the SL had higher fat mass compared to the other two groups. Even when corrected for differences in body weight, the over-nourished mice from the SL had higher body fatness compared to both, the NL and LL pups ($p < 0.01$ for both). Craig and Basset (20) demonstrated similar results in Wistar rats. In this experiment, the investigators redistributed pups to small, normal and large litters (4 pups:1 dam, 10:1 and

15 – 18:1 respectively). They observed that from postnatal day 3 until day 21, pups raised in the SL gained weight at a faster rate than the other two groups. For example, on day 13, the rate of gain in the SL pups was at least twice that of NL and LL pups (1.34 ± 0.15, 0.69 ± .13, 0.55 ± 0.11 g/day respectively) ($p<0.05$). These growth trajectories continued into adulthood, whereby, at 11 weeks of age, SL rats weighed 19% higher than NL and approximately 30% higher than LL rats ($p\leq0.01$).

Faust et al (21) examined whether the higher body weights were still observed in pups suckled in SL by 28 weeks of age. In this study, newly born pups were assigned to groups of four or twenty per suckling dam. All pups were weaned at 21 days to stock laboratory diet. The rats were allowed ad libitum access to diet and water until they were 28 weeks of age. Mean body weight of SL vs. LL rats were different at 749 ± 32 vs. 584 ± 16 g, respectively ($p\leq0.01$). Therefore, early under-nutrition had a lasting effect on lifelong growth trajectory. These studies demonstrate that the suckling period is one window of development whereby, nutritional intake can program life-long metabolism such that body weight in adulthood is increased.

3.1.2. Litter manipulation- changes in body fatness

Nutritional status during the early postnatal window can alter body fat content. In the rat, under-nutrition during the suckling/lactation period lead to reduced body fatness compared to well-nourished controls both, at weaning and in later life (22,23,24,25,26,). Stephens (27) demonstrated being suckled in a LL lead to a persistent reduction in weight and body fatness into adult life. In his experimental design, he distributed equal number of pups to the control and dams that were under-nourished during lactation. In order to

achieve sub-optimal nutrition, the lactating dam was fed 50% of matched ad libitum intake of the control dam. At 18 weeks of age, the pups that had been suckled on the malnourished dam weighed less than pups suckled on a control dam (309.0 ± 7.7 vs. 436.0 ± 11.1 g respectively) ($p \leq 0.001$). Body composition revealed that the under-nourished rats had less total body fat, both in absolute terms and as a percentage of body weight. For example, these rats had 13.6 ± 0.8 % compared to 18.4 ± 0.9 % body fatness observed in control rats ($p \leq 0.001$). Two possibilities arise for the differences observed between the two groups. First, the nutrient-deficient dam produced less milk; therefore, the pups consumed less milk during this period. As such, their appetite was programmed to continue to eat less in the post weaning duration and subsequently, they weighed less in later life. Unfortunately, the authors did not include food intake data to provide evidence for this inference. Second, the decreased body fat content of these rats compared to well-nourished controls may be due to decreased lipogenic ability resulting from less fat mass or vice versa.

Decreased body fatness resulting from under-nutrition was also reported in another study carried out by Faust et al (21). In this experiment, adult rats that were raised in the LL (20 pups per dam) weighed less and had less body fat, as measured by a composite of the major fat depots (epididymal, retroperitoneal and inguinal), compared to those raised in SL of 4 pups (10.9 ± 0.06 vs. 13.1 ± 1.8 g/100g body weight, LL vs. SL) ($p \leq 0.01$). In an attempt to understand the source of the observed difference, the authors determined fat cell number and size in adipose tissue. Animals in the LL group had a reduced number of fat cells compared to control animals ($p \leq 0.01$); however, cell size was similar between groups. Therefore, reduction in total nutrition during the suckling

window lead to alterations in adipocyte content, in this case, the number of fat cells available for energy storage.

Utilizing the litter manipulation method, Engerlbregt et al (26) noted that rats reared in LL (20 pups) grew more slowly and had less body fat mass than the ones raised in NL (6 pups). At 6 months, body weight of the restricted rat was 387.0 ± 16.4 g compared to 506.0 ± 19.6 g in the control group ($p \leq 0.01$). Using dual energy X-ray absorptionmetry, the authors quantified differences in body fat content and soft lean tissue. The rats that were raised in LL were leaner and had less body fat compared to controls. The mean percentage of body fat in the restricted group was 14.0 ± 2.0 compared to 19.0 ± 3.0 observed in the controls ($p \leq 0.05$). Lean soft tissue, as expressed relative to body weight, was 81.0 ± 2.0 % in the LL group vs. 76.0 ± 3.0 % in the NL group ($p \leq 0.05$). The authors theorized that one reason for the difference in body composition was that the LL rats were programmed to deposit more lean tissue compared to adipose tissue throughout life because lean tissue is more metabolically significant.

Conversely, over-nutrition during the suckling period results in larger gains in weight and higher body fat content (28,29,30,31,32,33,34). This observation was reported in Sprague Dawley pups over-nourished during the lactation period by suckling in SL (4 pups) as compared to those that suckled in LL (22 pups) (35). Body weight of the SL rats was heavier at 5 week post weaning in comparison to LL rats (192.0 ± 22.9 vs. 139.0 ± 13.0 g respectively) ($p \leq 0.01$). This trend continued until they were 20 weeks of age, at which time, the SL rats weighed approximately 200 g more compared to LL rats ($p \leq 0.01$). Body fatness as measured by epididymal fat pad weight was greater in the SL rats compared to LL rats starting at week 10 and persisted to week 20. The ratio

of fat pad to total body weight at 10 weeks of age in the SL rats was almost twice that of the LL rats (0.42% vs. 0.25% respectively) ($p \leq 0.05$). At 20 weeks of age, the SL rats continued to have a greater degree of fat/g body weight (0.87% vs. 0.39% in LL rats) ($p \leq 0.05$).

Similarly, Miller and Parsonage (36) noted that pups raised in SL comprising of 4 per dam were bigger at weaning and continue on this growth trajectory until 17 weeks of age, when these rats weigh approximately 120 g more compared to those raised in litters of 12 per dam ($p \leq 0.01$). The SL rats had higher carcass fat content at weaning and also at the end of the study. Using Pearson's correlation analysis, the authors found that at weaning, body weight had a strong and inverse correlation ($r = -0.8$) with litter size. In adulthood, the direction and magnitude of the correlation were still present. For example, the correlation between body weight and percent body fatness with litter size were $r = -0.9$ and $r = -0.8$ respectively. These relationships indicate that litter size during the suckling period had a strong and persistent negative association with the rate and direction of growth and gain in fat content.

3.1.3. Litter manipulation- potential mechanisms

The body of literature on the effect of altered nutrient intake during the suckling/transition period illustrates that this is one window of development whereby, changes in total nutritional intake are strongly associated with body weight and body fatness in later life. The potential mechanisms are many because energy balance is a composite of a multitude of events on different planes of metabolism. On the cellular level, altered lipid synthesis may be one mode leading to dissimilar fat deposition. On

the systems level, changes in neuroendocrine development may result in altered food intake and subsequently body weight.

Differences in the efficiency of energy metabolism may be one mechanism contributing to the observed differences in body weight and energy storage. Utilizing a litter manipulation model, Fiorotto et al (25) determined that Sprague Dawley rats raised in LLs (16 pups) were smaller and had less fat deposition compared to those raised in NLs (10 pups) because they were less efficient metabolically. In this study, milk intake of the pups was determined by measuring the difference between intake and output of tritiated water. On postnatal day 15, the pups reared in the NL weighed 34.5 ± 2.0 g whereas the LL pups weigh 25.1 ± 1.8 g. They also had 40% higher body fat content compared to the LL pups. Daily gross energy gain (as measured by energy gain divided by energy intake) of pups suckled in NLs was higher compared to LL pups (32.0 ± 3.0 vs. 26.0 ± 2.0%, respectively) ($p < 0.05$). In the long-term feeding study by Miller & Parsonage (36), the authors demonstrated that early over-nutrition lead to improved energy efficiency that persisted into adulthood. They calculated the net efficiency of each group based on the formula: [(gain in carcass energy/ (energy intake – energy cost of maintenance)] and found that adult rats previously raised in the SL group had improved utilization of their energy intake compared to rats in LL group (50.1 % vs. 22.6 % respectively) ($p \leq 0.05$). These two studies provide evidence to support a programming effect of early nutrition intake on energy metabolism, pointing toward an improved energy budgeting due to over-nutrition during suckling.

In contrast, another study examining this issue did not lend evidence for life-long programming of energy metabolism. Wiedmer et al (37) reported that although Wistar

rats raised in SL (2 pups) weighed more and had higher body fatness than those raised in NL (12 pups) at 5 and 12 weeks of age, energy metabolism, as calculated by differences in assimilated energy and energy expenditure was similar between the two groups. At 5 weeks of age, the SL rats had similar total energy intake compared to NL rats. Of the total energy intake, assimilated energy, calculated by subtracting the energy content of urine and feces from the energy consumed, was approximately 73.6 % for both groups. On the other hand, differences were noted in total energy expenditure (as measured by indirect calorimetry) between the two groups. At 5 weeks of age, SL rats expended 182.2 ± 5.0 kJ/day compared to 157.8 ± 4.3 kJ/day used by NL rats ($p \leq 0.005$). Applying the 73.6 % assimilated energy intake and daily energy expenditure values, the authors calculated energy balance and did not find significant differences in daily energy retention between the two groups (56.7 ± 8.5 vs. 56.2 ± 4.4, SL vs. NL). These data indicate that although the SL rats expended more energy, they ate a little more to compensate, leading to similar energy retention values between the two groups. The authors speculate that the increased body weight and fat mass in the SL rats at 5 weeks of age was due to preferential energy deposition into adipose tissue.

In order to study the potential programming of intrinsic energy metabolism from early postnatal over-nutrition independent of body weight at 12 weeks of age, these same authors used a group of NL pups that were weight-matched to a group of SL. This was achieved by hand-picking rats of similar weight from both SL and NL groups. They theorized that this method allowed them to bypass the possible confounding factors such as changes in body fat content, intake and expenditure associated with aging. Utilizing animals that were of the same weight between the NL and SL groups, the investigators

reported that daily energy intake and expenditure was not different, thus energy retention was also the same between the diet groups. It should be noted that the authors used the same energy assimilation percentage observed at week 5 in their calculation of energy retention at week 12. It would have been helpful to determine the percent of assimilated energy at week 12 in order to separate out potential differences in energy utilization in young adulthood. Furthermore, the authors' objective was to determine the possible persistent re-programming effects of early dietary modification on later metabolism. By controlling for differences in body weight at 12 weeks of age, the investigators were able to determine the intrinsic differences of energy metabolism at this age. However, in the context of programming, this technique could have blurred the composite programming effects of early over nutrition.

These studies provide evidence that the early nutritional experience can imprint energy efficiency. Although the duration of dietary change required to imprint metabolism needs further investigation, over-nutrition during the suckling period may improve energy deposition into adipose tissue.

Another theory to explain the changes in adiposity induced by nutrient modification in early life explores the processes of cellular development. Winick and colleagues hypothesized that cellular growth encompasses two stages: cell division and cell enlargement. Evidence from a sequence of studies utilizing rat pups that were under-nourished in the suckling to weaning transition suggested that cellular effects of under-nutrition may differ (38,39,40). In the study done by Knittle & Hirsch (35), they noted that the increased weights of the epididymal fat pads in the SL rats was due to increased number and size of adipocytes at 5 weeks and 20 weeks of age. For example, at 20

weeks, rats raised in SL had approximately twice as many cells in the fat pads, and these cells were twice as large as adipocytes found in the fat pad of LL rats ($p \leq 0.01$ for both). A similar study done in mice, but lasting for a longer duration reported that although hypertrophy was noted at 13 weeks in mice that were over-nourished in the suckling period, this difference was no longer significant by 52 weeks (31). The authors suggested that in the mice under nourished during suckling, cell enlargement may have occurred later in life, between 19 and 26 weeks, thus, by 52 weeks, cell size had caught up with that in SL males, where cell enlargement occurred earlier in life.

On the other hand, Sprague Dawley pups raised in litters of NL (9 pups) and allowed ad libitum intake until 43 weeks of age grew faster and had larger fat pads compared to those raised in LLs (24 pups) by the end of the 43rd week ($p \leq 0.02$ for both) (41). In the NL rats, both, cell size and cell count tended to be higher compared to LL rats but did not reach statistical significance ($p > 0.07$for both). However, in comparison to the rats that were raised in SL (4 pups), the LL rats had significantly more adipose cells and these were larger ($p < 0.05$ for both) at the end of the 43 weeks. Therefore, these studies generated two points. First, adipocyte size may have a set volume limit. As such, restricting nutrition intake may result in under-development in the early phase of growth. However, long-term ad libitum intake can modify early imprinting, whereby cell size development 'catches-up' in the long-run. Therefore, ad-libitum intake throughout the lifespan may ablate the effects of early nutritional restriction. Second, the severity of nutritional modification can impact the extent and duration of the programming effect.

Thus far, accumulated evidence illustrates that in the newborn rodent, over or under-nourishment by litter manipulation modifies growth and fat deposition. In terms of

fat accretion, the length of the programmed effect remains to be clarified. Because of the uncertainty, some authors have measured biochemical parameters to try to account for these differences. In Wistar rats over fed due to suckling in SLs, hypertrophic adipocytes were noted at 2 weeks of age in comparison to age-matched controls ($p \leq 0.001$), however, by 8 weeks of age, this difference was no longer significant due to enlargement of cells in the control group between 2 and 8 weeks (29). Levels of lipoprotein lipase (LPL) activity was higher in the in the over fed pups between 1 and 2 weeks of age in comparison to controls, but this did not continue into the 8th week of the study. Hyperinsulinemia and increased plasma triglyceride (TG) levels ($p \leq 0.01$ for both) were also observed between the 1st and 2nd week of life in the SL pups, although these differences were not observed at 8 weeks. This temporal pattern suggests that increased TG substrate and higher insulin concentrations may act in concert to stimulate LPL activity, thus leading to increased adipocyte growth in the early life.

Longer periods of imprinting have been reported by other investigators. Hahn (28) noted that hyperinsulimenia observed in over-nourished rats raised in litters of 4 was evident at 7 weeks of age. Similarly, Cryer & Jones (42) observed persistent increases in insulin levels in overfed rats suckled in litters of 4 compared to those raised in litters of 16. LPL levels were also elevated at 14 weeks of age and continue to 44 weeks in the SL rats. These authors did not observe differences in cell number between the two treatments; however, cells in the SL rats were larger compared to those raised in LLs. Although there are temporal differences between the studies, the increase in adipocyte content along with higher LPL activity and higher insulin levels suggest that early dietary

modification can have an effect on adipocyte metabolism; however, the time of onset and duration of change needs further investigation.

Another way in which early nutritional intake may promote obesity in later life is through a lasting impact on the central nervous systems (CNS) that control food intake, satiety, or both (43,20). McCance, in 1976 (44) put forth the idea that nutritional conditions during the suckling to weaning transition can permanently alter the hypothalamic center of appetite control. Thus, rats with high food intake early in life will continue to have high intakes later in life, while those with restricted intakes will consume less food later in life. This theory was supported by work from Harris (43), whereby he demonstrated that adult rats raised in SL (3 pups) had higher food intake throughout life, until 20 weeks of age, compared to those raised in LL (16 pups). Similar results were reported by Oscai & McGarr (45) in which rats raised in LLs voluntarily consumed less in the post-weaning period when given ad libitum access to food. Thus the LL pups grew slower and gained less fat compared to those raised in SLs. This difference was noted from weeks 4 to 61, such that during the 58 weeks that the data was collected, the rats raised in LL consumed an average of 1,620 g less chow than those raised in SL ($P<0.001$). Therefore, in these animals, there seem to be a re-programming of intake due to early under-nutrition.

This is in contrast to the findings of Lambert & Doeslag (46). These authors demonstrated that animals suckled in LL weigh less than those suckled in SL and NL from weaning to 18 weeks of age ($p \leq 0.05$). However, when weaning weight was used as a covariate, the differences in body weight were no longer significant. Body fatness measured relative to body weight was also not different between litter sizes.

Furthermore, food intake during this time was not affected by early dietary restriction. The authors noted the discrepancy between their results and those of others, and attributed the differences to housing conditions. After controlling for litter size and gender, mean food energy intake in rats housed as pairs were significantly higher than those housed singly (440 ± 14 vs. 403 ± 14 $kJ.day^{-1}$). Therefore, this study points out the importance of environmental conditions and social behavior that may affect outcome parameters. Thus, other factors such as housing conditions may override possible programming effects resulting from early nutritional experiences.

Although the phenomenon of increased weight gain resulting from early over-nutrition is well-documented, mechanisms contributing to these results need further clarification. It has been demonstrated with certainty that rodents raised in SL during the suckling period develop faster and have higher body fat content compared to those raised in LLs. Direct measurement of food intake during this phase of the lifespan is difficult, and as such, there are few studies in this area. Nonetheless, one investigation reported that acceleration of growth and fat deposition due to over-nutrition during suckling is a result of increased ingestion of milk by pups raised in smaller litters (25). Some researchers theorize that this may re-program neuroendocrine development toward higher food intake habits that persist into adulthood. Two centers in the brain that are thought to control satiety are the hypothalamic arcuate nucleus (ARC) and the paraventricular nucleus (PVN) (reviewed in ref 47). Neuropeptide Y (NPY) innervation from the ARC during fasting and other states of negative energy balance stimulates feeding behaviour (48) and injection of NPY into this region result in hyperphagia and weight gain (49). Plagemann and colleagues have demonstrated through a series of experiments that over

nutrition during the suckling peroid alters brain messaging along with hormones relating to intake and satiety. In Wistar rat pups over-nourished by litter reduction (3 pups), a significantly increased body weight was observed compared to pups in NLs (10pups) ($p \leq 0.001$) (50,51). Radioimmunoassay for NPY levels in the brain of 21 day old over-nourished rats revealed that they had similar NPY content in the ARC compared to NL or LL pups (SL: 35.0 ± 2.2 vs. NL: 38.0 ± 1.9 vs. LL: 47.0 ± 4.6 mg/mg). However, when NPY-positive neurons were expressed as a percentage of the total number of neurons, SL rats had significantly higher content of NPY+ neurons in the ARC (SL: $45.0 \pm 0.8\%$ vs. NL: $39.0 \pm 1.4\%$) ($p \leq 0.005$). Interestingly, concomitant hyperleptinemia and hyperinsulinemia were observed in the SL pups. The mean leptin level in SL rats was 12.0 ± 1.3 vs. 3.9 ± 0.5 ng/mL in the NL group ($p \leq 0.001$) and mean insulin level in SL rats was 43.0 ± 6.8 vs. 21.0 ± 3.8 mIU/L in the NL rats ($p \leq 0.01$) (52). Both, insulin and leptin act as adiposity signals by inhibiting NPY neurons and high levels of these hormones produce anorexic responses including decreased food intake (reviewed in ref 53). Although we would expect an inhibitory effect, this was not the case as evidenced by larger gains in weight in the SL pups. Since the NPY system is functionally mature by 21 days in rats, the authors speculate that there may be early signs of insulin and leptin resistance in these pups. It should be noted that in later studies, Plagemanns's group demonstrated that insulin can exert an inhibitory effect on the neurons of the SL pups. For example, in *in situ* conditions, administered insulin decreased the rate of firing of neurons in the hypothalamic nucleus from the SL pups more than neurons from the NL pups ($p \leq 0.01$) (54). They also demonstrated that prophylactic leptin treatment in the SL pups did not result in decreases in body fat content (55). These results suggest that in

young over-fed rats, early aberrant peptide and hormone signaling may occur, thus contributing to altered food intake and weight gain.

Two extensions to these studies involved investigation of the responsiveness of neurons to orexigenic neuropeptide NPY and anorexic peptides, cholescyctokinin (CCK) and leptin. CCK is released from intestinal endocrine cells during food intake. The general process follows that CCK binds with CCK-A receptors on gut vagal efferent nerves that end in the brainstem leading to the PVN. Sufficient innervation leads to cessation of meal intake (56). In 21 day old SL rats (6 pups) that were heavier compared to NL rats ($p \leq 0.001$), there were fewer CCK-positive neurons in the PVN region compared to NL rats ($p \leq 0.002$) (57). Unfortunately, administration of CCK did not produce significant differences in neuronal activation between the SL and NL pups. On the other hand, neurons in this region of the brain of 21 day old SL rats were more responsive to NPY administration compared to neurons of NL rats ($p \leq 0.01$). Therefore, in the case of NPY, increased inhibition of these neurons in SL rats can be interpreted as an increased effectiveness of the action of NPY in attenuating the normal negative feedback loop that controls satiety (58). These results demonstrate that alterations in the early dietary experience during a critical phase of neuroendocrine development may lead to alterations in intake and subsequent weight gain. Future investigations with longer study durations may provide insight into the underlying mechanisms contributing to altered habitual intake in the post weaning period.

3.2. Early introduction of carbohydrate - the artificial rearing (AR) model

The majority of postnatal dietary modification in animals utilizes the litter manipulation methodology to modify total nutrient intake. The suckling period poses special technical challenges in terms of experimental design because of the particular nature of delivering milk and caring for vulnerable newborn pups. In 1975, Hall (59) modified an existing artificial feeding method designed by Messer et al (60). This enabled researchers to alter the nutritional content of milk ingested by pups during the suckling period. Patel's group has adapted this method to investigate the effects of high carbohydrate (HC) intake from 4 to 20 days postnatal on glucose metabolism and growth. Briefly, pups are reared for 20 days via intragastric feeding of a substitute milk formula consisting of 56% carbohydrate, 24 % protein and 20% of calories from fat. In comparison, rat milk contains 8% carbohydrate, 24% protein and 68 % fat. This method was used in all their studies with minor modifications that will be mentioned during the discussion of each paper.

In one of their earliest works, the authors utilized the AR method and weaned the pups to a rat chow until 9 weeks of age. At this point, they fed the rats a high sucrose (52% sucrose, 15% cornstarch, 21% protein and 12% fat) diet until 14 weeks of age (61). They observed that pups fed the HC formula during suckling had perturbations in lipid synthesis, larger fat mass and increased body weight in adulthood. Although body weight was not different during the suckling period, at approximately 7 weeks of age, the HC rats started to grow more quickly than suckle control rats and by 14 weeks of age, the HC rats were 133 g heavier than suckled rats ($p \leq 0.01$). At this time, liver and epididymal fat pads were also larger in the HC group: (liver: 28.2 ± 1.6 vs. 21.6 ± 1.6 g,

fat pad: 16.9 ± 1.1 vs. 12.9 ± 0.4 g, HC vs. control) ($p \leq 0.01$ for both). In adult HC animals, cells in epididymal tissues were 70% larger compared to control animals ($p \leq 0.005$). At 7 weeks of age (during the chow fed stage) non-fasting insulin levels in the HC rats were two-fold higher compared to control rats ($p \leq 0.001$) as well as at 14 weeks of age (after 5 weeks of high sucrose intake) ($p \leq 0.001$). Lipogenic capacity of both, liver and adipose tissues were enhanced in the HC group as measured by an increased incorporation of ^{3}H fatty acid into these tissues ($p \leq 0.01$). In adipose and liver tissues, fatty acid synthase activity was higher in the HC rats compared to controls, and glucose 6-phosphate dehydrogenase activity was also higher in adipose tissue of HC rats. These findings demonstrate a persistent effect of HC intake during suckling on metabolism leading to increased body weight in adulthood.

To determine the potential mechanisms that may explain the observed effects, Patel and his group examined the hormonal and biochemical characteristics of rats fed the HC formula. During the HC intervention, body weight did not differ between rat pups in the suckle control and those in the HC groups; however, a number of immediate biochemical and cellular changes were noted during this period. At 12 days of age, the HC rats had a six-fold increase in circulating plasma insulin levels compared to the suckling rats (382 ± 87.0 vs. 61 ± 4.0 pM) ($p \leq 0.01$) (62). Despite the marked increase in insulin, circulating plasma glucose was similar between groups (6.5 ± 0.1 vs. 6.7 ± 0.2 pM). In order to tease out the source of hyperinsulinemia, the investigators isolated islets and incubated them in four glucose concentrations (1, 2.8, 5.5 and 16.7 mM) for either 10 or 60 minutes. At both time intervals, the islets from HC rats secreted significantly more insulin compared to those isolated from pups in the suckle control group. For example,

after 10 minutes at 5.5 mM glucose, the islets isolated from the HC rats secreted 15-fold more insulin compared to islets from the suckle control group ($p \leq 0.0005$). Therefore, these findings suggest that early introduction of a HC diet resulted in increased insulin secretion, contributing to hyperinsulinmenia. On the cellular level, immunohistochemistry revealed an increase in islet number in the pancreas of the HC pups along with a larger amount of area occupied by β-cells in the pancreas (63). Furthermore, the process of insulin synthesis was enhanced due to increased pre-proinsulin gene transcription activity (64). They also noted that enzymes in the glycolytic pathway were increased in the HC pups compared to suckled pups (62). Results from this research group indicate that HC intake during the suckling period leads to adaptations in the pancreas resulting in hyperinsulinemia as measured *in vivo and in vitro* methods. These alterations seem to re-program insulin-stimulated glucose utilization and are associated with changes in energy metabolism and weight gain in adulthood. It should be noted that Patel's AR method does not include a control group for the AR process itself. Therefore, we can't conclusively determine whether the effects of HC are due solely to HC metabolism or HC formula fed via the AR technique. Stressors relating to the AR process could have impacted outcome measures, and to what degree, is uncertain. Therefore, future AR studies should include a control group to remove this uncertainty.

The results in both the litter manipulation and artificial rearing studies provide evidence of programming from postnatal dietary modification. At the present time, the sensitive developmental window has not been established. Nonetheless, the suckling period may be one phase of growth that is susceptible to alterations in nutrient intake.

4. Trends in fructose consumption

Over the past two decades, dietary patterns of children have changed, whereby consumption of sweetened beverages has increased (65,66). The concomitant increase in childhood obesity coinciding with increased intake of FR sweetened beverages raises the question of whether this association may be part of a causal pathway to obesity and has prompted investigation in this area (67). In a prospective study, Ludwig et al (68) surveyed 548 ethnically diverse schoolchildren in a Massachusetts community from 1995 to 1997 to determine sweetened beverage consumption patterns. Regression analysis revealed that for each additional serving of sugar sweetened drinks consumed per day, the odds of becoming obese increased 1.6 times. One theory is that compensation for energy consumed in liquid forms is less than energy intake from solid sources (69). However, sugar sweetened drinks contain a high percentage of FR in the form of high fructose corn syrup (HFCS). For example, two 12 oz soft drinks contain about 50 g of fructose (~200 kcal) (70). Currently, HFCS is the preferred sweetener in the food industry and is used extensively in our food supply (71). Examples of other foods containing HFCS that are consumed on a daily basis are: breakfast cereal, snacks, condiments, and commercial baked goods. Based on food disappearance data, the per capita, daily FR consumption increased from 40 g/day in 1977-78 to 97 g/day in 1997 (7).

There are two sources of dietary FR, naturally occurring and added FR. Naturally occurring sources are derived from FR and sucrose (½ FR and ½ glucose) in fruits and vegetables, honey and grain products. Added FR comes from FR-containing sweeteners used in food processing. During the 1970s, HFCS became the preferred sweetener over sucrose (made from sugar cane and beets) for commercial applications due to its

functional characteristics and lower cost (71). For example, FR can enhance fruit, caramel and chocolate flavours in food. HFCS is also considerably less expensive to manufacture than sucrose due to abundant corn supplies and subsequent lower corn prices as compared to sugar cane or sugar beet prices (6). As a result, it is used extensively in carbonated beverages, commercial baked goods, breakfast cereals and canned fruits (71). In the US, in 1985, HFCS accounted for about 35% of the total amount of sweeteners used in food processing (7). By 1997, beverages alone accounted for 72% of the total HFCS used by the foods and beverages industry (6). Although Canadian data is not available to document current FR utilization rates in the domestic food market, the per capita consumption of sweetened beverages and foods have increased within the last decade (72). Therefore, it can be inferred that the amount of FR used in our food supply will continue to grow.

Based on 1977-78 USDA Nationwide Food Consumption Survey, the average American adolescent or adult consumed about 40g of FR per day, of which, about 13 g/day came from naturally occurring FR and 27g/day from FR added during processing (7). Due to lack of current statistics regarding consumption of FR, food disappearance data has been used to estimate the per capita intake of HFCS (6). By 1997, the approximate daily intake of FR increased to 97g/day. Within this quantity, consumption of FR from naturally-occurring sources remained relatively stable, while added sources of FR increased to 82 g/day (6). Separating daily intake into age/sex categories reveals that adolescents consume the highest amount of FR with young males (15 – 18 y of age) eating about 100g/day (7). Between 1965 and 1996, consumption of soft drinks by adolescents increased by 155% (65) coinciding with increased rates of childhood obesity.

4.1. Fructose metabolism

The majority of ingested FR is transported from the small intestine via the hepatic portal vein into the liver (73). In humans, approximately 50% of FR is metabolized by the liver (74), 20% via the renal system and the remaining 30% is disposed by adipose and muscle tissues (75). FR is a ketohexose that is metabolized slightly different than glucose. Upon entering the liver, it is phosphorylated by fructokinase using ATP to form FR 1-phosphate (76). Fructokinase has high specificity for FR; therefore, the liver is able to extract high amounts of FR from the blood (73). Aldolase B then cleaves FR 1-phosphate into dihydroxyacetone phosphate and glyceraldehyde. These two intermediates enter glycolysis as trioses and can be converted to glyceraldehyde-3-phosphate.

The difference between FR and glucose metabolism lies at this critical point. FR bypasses the enzyme phosphofructokinase, which is the main rate-limiting enzyme in glycolysis, and as a result misses this key feedback mechanism (77). The large quantity of glyceraldehyde 3-phosphate leads to elevated production of pyruvate and lactate. In contrast, hepatic glucose metabolism is inhibited by feedback to phosphofructokinase by the accumulation of citrate and ATP (70). The high concentration of circulating pyruvate produces large amounts of acetyl-coA. This molecule has three roles in the body; it can either be converted to carbon dioxide through the Kreb's cycle or form ketone bodies. However, of interest is its contribution to lipogenesis by forming long chain fatty acids. Uncontrolled production of dihydroxyacetone phosphate can lead to increased quantities of very low-density lipoprotein (VLDL). The function of this lipoprotein is to transport

triglycerides from the liver to peripheral tissues and high levels of VLDL have been implicated in coronary heart disease (78).

4.2. Fructose consumption and body weight

Currently, the available literature on FR metabolism examines the immediate effect of high FR intakes on changes in metabolic profile. In humans, there are few studies investigating the effect of high FR diets on weight gain. Tordoff et al (79) performed a 3 weeks study where 30 normal weight subjects consumed regular meals ad libitum, while being supplemented everyday with 1150 g soda sweetened with either artificial sweetener (AS) or HFCS. When both sexes were combined, the HFCS group had a significant weight gain while the AS group experienced a weight reduction. However, the sex of the subjects had an impact on the results. For example, females who drank the HFCS sodas had a significant weight gain of 0.97 ± 0.25 kg ($p<0.05$ vs. AS), while the males did not experience similar increases in body weight. In another study, 40 overweight men and women supplemented their diets with either a high amount of sucrose or AS for 10 weeks (80). The sucrose group had an average weight gain of 1.6 kg, of which 1.3 kg was a gain in fat mass. The AS subjects lost an average of 1.0 kg each person. It should be noted that both of these studies were designed to test the effect of FR or sucrose versus AS on weight gain. Therefore, the increased weight from either FR or sucrose could be attributed to increased caloric intake of these sugars compared to the low caloric nature of AS. For example, in the second case, sucrose supplied an average of 800 kcal/day whereas AS provided only 230 kcal/day (80). Also, researchers of both studies found that subjects who consumed the non-AS diets significantly increased their ad libitum energy intake and subsequently gained weight. For instance, in

the Tordoff et al. study, the AS group decreased their ad libitum food intake significantly by 7% which is in marked contrast to the 13% increase in calories ingested by the HFCS group (79).

To investigate the effect of FR versus starch on weight gain in subjects with diabetes, Anderson et al (81) had 14 middle-aged men with diabetes consume either a control diet (40% of energy from starch and 15% from non-FR sugars) or a FR-enriched diet (30% of energy from starch, 10% from FR, 15% from other sugars). Both diets provided 55% of energy from carbohydrates. The FR group increased their energy intake and subsequently gained an average of 1.9 kg within 24 weeks.

In animal studies, the evidence is unclear as to the effect of high FR consumption on weight gain. Hallfrisch et al (82) found that rats that were fed a high fat diet (40% of total energy) containing sucrose (30% of total energy) had significantly higher body weight and larger epididymal and perirenal fat pads compared to those that consumed a high fat diet with 30 % starch. Body weight of the sucrose group was 475 ± 7.0 g whereas, the starch group weighed 444 ± 5.9 g ($p \leq 0.005$). After correcting for differences in body weight, the sucrose-fed rats still displayed increased body fatness compared to the starch-fed rats ($p \leq 0.01$). Kasim-Karakas et al (83) demonstrated that hamsters on a high FR diet became obese compared to those on the control or high sucrose feeding. Both of the later groups had similar daily food intakes (11.1 ± 0.6g/day for sucrose and 8.3 ± 0.8 g/day for controls), whereas, the FR-fed animals consumed significantly more food (26.5 ± 2.8 g/day). Consequently, the FR group weighed more than both, the sucrose and the control groups (155± 5.3, 136 ± 7.2, 131 ± 7.5 g, respectively) ($p<0.05$). In order to determine if drinking FR in an aqueous form that is

similar to soft drinks might promote obesity, Jurgens et al allowed 3 month old mice ad libitum access to one of three solutions in addition to ad libitum feeding of standard chow for approximately 70 days (84). The solutions are as follows: 15% FR dissolved in water, 10% sucrose in water and a non-caloric "diet" soft drink. Water served as the control group. At the end of the 10 weeks, the FR group gained more weight than all other groups (47.9 ± 1.4, 44.0 ± 0.7, 43.6 ± 1.3, 44.0 ± 1.5 g, respectively) ($p<0.05$). Utilizing nuclear magnetic resonance to determine body composition, the authors observed increased body fatness in the FR group compared to the other diet treatments (10.5 ± 2.5, 7.9 ± 1.0, 7.4 ± 1.6, 5.4 ± 1.5%, respectively) ($p< 0.05$). Interestingly, the mice that drank the FR solution ate less chow during the 10 week period than the other groups, therefore, other mechanisms beside food intake promoted the increased weight gain in the FR-mice.

However, other investigators have reported the opposite effects of FR metabolism on body weight in animals. In a study to test the effect of different diets on eating patterns, 344 Fisher rats at the age of 3 months were assigned to either a standard rat chow, high FR or high glucose diet for 10 days (85). The researchers found that the rats on the FR-supplemented diet ate significantly less compared to the controls or the glucose group (16.8 ± 0.9, 19.9 ± 0.7, 19.6 ± 0.9 g/day, respectively). As a result, the FR fed rats had smaller gains in body weight compared to the rats on the other diets. Similar results were reported for a 12 weeks study where 24 days old rats were fed either a FR-enriched or starch diet (86). The FR diet caused the rats to grow more slowly than those fed a diet rich in starch. At the end of the 12 weeks, the FR group had gained 94 g, while the starch group had gained 113 g. From the available studies, the effect of FR intake on

body weight remains inconclusive in both humans and animals. However, many of these studies were not designed specifically to test the effect of dietary FR on weight gain; therefore, it is important to look at the metabolic consequences of FR to try to understand its effect on body weight.

4.3. Fructose intake and insulin

Insulin has a key role in regulating energy balance by its action on insulin-sensitive tissues such as skeletal, liver, adipocytes and brain cells (reviewed in 87). It is generally accepted that circulating insulin crosses the blood-brain barrier into the CNS where it attaches to insulin receptors to produce anorexic responses, including reduced food intake (reviewed in 88). Bruning et al. worked with mice to produce disruptions in the insulin receptor gene in the neurons of the CNS. These NIRKO (neuron-specific insulin receptor knockout) mice were hyperphagic and had high amount of body fat and developed obesity (89).

Insufficient insulin production can also hinder signals to reduce energy intake. FR intake produces less insulin to be released compared to glucose. Infusion of FR into rat pancreas at 200 mg/dl did not stimulate insulin secretion. However, the same amount of glucose infusion resulted in a significant increase in insulin release from pancreatic beta-cells (90). Grant et al. found that incubation of isolated islets of Langerhans with glucose resulted in insulin production while this effect was not seen when the islets were incubated with FR (91). Similarly, Havel found that insulin levels increased after an intravenous glucose infusion, whereas FR infusion did not stimulate insulin secretion (92). Insulin plays an important role in regulating energy homeostasis by providing a

negative feedback loop to inhibit food intake. Because FR is less likely to stimulate insulin release, less insulin is actually binding with the receptors in the CNS to signal a reduction in intake. As such, the amount of energy intake is not regulated in the short-term.

Chronic consumption of high dietary FR can lead to insulin resistance. This may be another factor contributing to distorted energy homeostasis. Insulin resistance is characterized by a reduction in the amount of insulin suppression of hepatic glucose production and/or reduced insulin stimulation of glucose uptake into muscle and adipose tissue (93). This has implications for long-term energy regulation. High FR feedings caused insulin resistance in dogs (94) and rats (95). Tobey et al. found that chronic administration of FR in rats lead to hyperinsulinemia and *in vivo* insulin resistance (96). They suggest that insulin resistance is due to the diminished ability of insulin to lower endogenous glucose output. Similarly, Pagliassotti and Prach found that both a low sucrose diet (18% of total energy) and a high sucrose diet (68% of total energy) resulted in impaired suppression of hepatic glucose production leading to insulin resistance in young rats (97).

Although the above researchers theorize that insulin resistance is due to higher hepatic gluconeogenesis, it can also occur through impairment in muscle uptake of glucose by insulin. Kim et al. (98) demonstrated that knocking out muscle insulin receptors in mice caused them to gain a large amount of fat mass. They suggest that insulin resistance can change the distribution of substrate from muscle to adipose tissue, thereby contributing to adiposity. Insulin resistance is of importance in high FR

consumption because it impedes the liver's ability to control endogenous glucose production or it can lead to less utilization of the available glucose by muscle.

4.4. Fructose intake and lipids

In animal models, dietary FR consistently results in hyperlipidemia (reviewed in 70). The evidence in human studies is less clear. In a comprehensive review (99), Hollenbeck analyzed the results of 18 investigations on the effect of FR and lipids, and concluded that high dietary FR significantly increases fasting triglyceride levels. Furthermore this effect was demonstrated at consumption rates that reflect the typical Western diet (7.5% - 20% of energy).

The mechanism of FR metabolism can be traced to the liver, as it is the key organ for disposing of FR (74). This sugar is more readily incorporated into fatty acids than glucose (100,101) and FR metabolism results in lipogenic intermediates G3P and pyruvate. Through esterification, G3P is changed to acyl glycerol, and with the addition of cholesterol becomes VLDL. In a review by Parks and Hellerstein (102), the authors concluded that high carbohydrate diets lead to elevated triglyceride levels, and this is due in part to increased VLDL secretion by the liver. High FR intake also increased the amount of plasma nonesterified fatty acids (103). These bind to serum albumin for transport to muscle or liver tissue for oxidation or esterification to make VLDL (104).

Intravenous injection of FR (at either 200 mg or 400 mg) into anaesthetized rats yielded high G3P and pyruvate concentrations (105) whereas, glucose injection did not produce the same results. Burch et al. demonstrated increased levels of hepatic pyruvate and G3P when FR was administered into the intraperitoneal area of rats (106). When perfused rat livers were infused with FR, secretion of VLDL triglycerides and

esterification of free fatty acids increased. This effect was not observed with glucose infusion (107). In animal studies, FR consumption has been correlated with hyperlipidemia, but in humans, this correlation is not quite as clear. Although the available research indicates that FR metabolism can have negative effects on lipid profile.

5. Summary & rationale

In North America, the prevalence of overweight and obesity have increased dramatically within the past decade, and it is of special importance as childhood obesity continues to rise. Dietary patterns are one factor contributing to weight gain. In the past, high fat consumption was implicated in causing increased body weight. However, on a national level, fat intake has decreased over the past decade, while the incidence of obesity continues to climb. FR consumption rose in the past two decades due to its wide application in commercial products such as soft drinks and sweetened food products.

FR metabolism differs from glucose and as a result, stimulates less insulin production. However, chronic FR feeding in animals leads to hyperinsulinemia and insulin resistance causing distorted energy balance. Due to the lipogenic nature of FR digestion, chronic intakes of this sugar result in VLDL triglyceride production that can lead to hyperlipidemia. At the present time, the effects of FR ingestion on body weight are conflicting.

Evidence from the metabolic programming literature demonstrates that the suckling to weaning transition may be one sensitive developmental period whereby nutritional modifications can alter metabolism. It has been illustrated with certainty that pups that were over-nourished from being raised in small litters weigh more and have

more body fatness compared to those that were suckled in normal or large litters. Interestingly, these growth trajectories continue into adulthood without further dietary treatments; suggesting a persistent programmed response from the early dietary experience. Currently, the underlying mechanisms require further investigation. However, there may be changes in enzymatic and hormonal control of adipose development. Work by Patel's group demonstrates that high carbohydrate intake during the suckling period result in altered insulin activity, possibly due to changes in β-cell morphology and function.

Combining the two bodies of literature, we hypothesize that early introduction of FR, specifically during the suckling phase of growth, will result in increased body weight in adulthood via programmed response.

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Chapter 2: Results from cohort #1

Dietary Fructose during Suckling Increases Body Weight and Fatty Acid Uptake into Skeletal Muscle in Adult Rats

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1. Introduction

Nutritional intake during sensitive developmental stages can influence health status in later years (1,2). This concept, known as the Developmental Origins of Health and Disease hypothesis, has received considerable attention by investigators studying how compromised maternal nutritional intake during gestation may "program" the offspring's metabolism so that these offspring have increased risk of obesity, cardiovascular disease and diabetes in adult life (3,4,5). The effects of alterations in nutritional intake during the early postnatal period on risk for chronic disease in later life have not been well delineated, particularly in the context of dietary changes observed during the past 2-3 decades.

Two methods that have been used to manipulate the early postnatal diet in animal models are litter size manipulation and artificial rearing (AR) of pups. The first, litter size manipulation allows investigators to change the total caloric intake of pups during the suckling period by redistributing them into various suckling litter sizes (6). Data generated from this model has demonstrated that rodents raised in small litters (SL) of 4 – 6 pups per dam weighed more at weaning and continued on an accelerated growth trajectory until adulthood compared to those raised in large litters (LL) of 15 or more pups per litter (7,8,9,10,11,12). Aubert et al (13) observed that mice suckled in SL were heavier compared to those reared in LL at 28 days of age. This growth trajectory continued until they were 18 weeks of age, at which point, the SL mice weighed 13 grams more compared to LL mice ($p<0.01$). Similar results were demonstrated by Bassett & Craig (9) whereby, they reported that from postnatal day 3 until day 21, pups

raised in SL gained weight at a faster rate than the other two groups. For example, on day 13, the rate of gain in SL pups was at least twice that of control and LL pups ($p<0.05$). This growth pattern persisted into adulthood, and at 11 weeks, SL rats weighed 19% more than controls and approximately 30% more than LL rats ($p \leq 0.01$). Therefore, these studies demonstrate that dietary manipulations during the suckling period of growth can have a lasting effect on metabolism such that long-term growth is affected.

Because the suckling period is one key window of development, other investigators have developed an artificial rearing method in order to manipulate specific dietary components during this time. In this model, gastric cannulas are used to deliver formula. Patel and colleagues have used the AR technique to explore the effects of high carbohydrate (HC) introduced during suckling on glucose metabolism. In one study, they randomly assigned Sprague-Dawley pups to nurse on a dam or artificially reared from days 4 to 12 postnatal (14). The macronutrients in the milk formula consisted of 56% of energy as carbohydrate, 24 % as protein and 20% as fat. In comparison, rat milk contains 8% of energy as carbohydrate, 24% as protein and 68 % as fat (15). At 12 days of age, the HC rats had a six-fold increase in circulating plasma insulin levels compared to the suckling rats (382 ± 87 *vs* 61 ± 4.0 pM) ($p \leq 0.01$). Despite the marked increase in insulin, circulating plasma glucose was similar between groups, thus indicating hyperinsulinemia. In order to tease out the source of hyperinsulinemia, the investigators isolated islets and incubated them in four glucose concentrations (1, 2.8, 5.5 and 16.7 mM) for either 10 or 60 minutes. At both time intervals, the islets from the HC rats secreted in a dose dependent manner, significantly more insulin compared to those isolated from suckling rats. For example, after 10 minutes at 5.5 mM glucose, these islets

secreted 15-fold more insulin compared to control islets ($p \leq 0.0005$). Therefore, these findings demonstrate that early introduction of HC resulted in hyperinsulinmenia.

To test the possibility of programmed effect of early HC feedings, Patel's group used the similar AR design, but extended the experimental period to 100 days (16). In this study, the AR rats were fed through the gastric tubes from 4 to 18 days postnatal, then fed via feeding tubes from 18 to 24 days postnatal. At 24 days, both groups were weaned to stock laboratory diet until 100 days of age. Rats fed the HC milk formula early in life gained more weight compared to suckled rats (603 ± 8 vs 434 ± 8.2 g) ($p \leq 0.001$). Hyperinsulinemia observed at 12 days of age in the HC group continued into adulthood (1195 ± 46 vs 342 ± 8 pM, HC vs suckling groups) ($p \leq 0.001$), whereas, both groups continue to be euglycemic. These findings suggest two things. First, that early introduction of HC can have a lasting effect on metabolism, thus promoting weight gain in later life. Second, suckling is one plastic developmental period where dietary manipulations can affect lifelong development.

Over the past two decades, dietary patterns of children have changed. One such trend is the increased consumption of fructose (FR), often via increased intake of sweetened beverages containing high fructose corn syrup (HFCS) (17, 18,19). The concomitant increase in childhood obesity coinciding with increased intake of FR has prompted investigation in this area (20). In a prospective study, Ludwig et al (21) surveyed 548 ethnically diverse schoolchildren in the Massachusetts community from 1995 to 1997 to determine sweetened beverage consumption patterns. Regression analysis revealed that for each additional serving of sugar sweetened drinks, the odds of becoming obese increased 1.6 times. Currently, HFCS is the preferred sweetener used in

the food industry and thus, it is prevalent in our food supply (22). Examples of other foods that are high in HFCS include breakfast cereal, snacks, condiments, and commercial baked goods.

Unlike glucose, acute ingestion of FR does not stimulate insulin secretion (23,24). However, it has been documented in rodent feeding studies that chronic feeding of a high FR diet induces glucose intolerance, insulin resistance, hyperlipidemia, and in some instances, hypertension (25,26,27,28). Current literature points to an intertwined mechanism of perturbed lipid metabolism, possibly in the form of increased fatty acids, on insulin sensitivity (29). Utilization of free fatty acid encompasses a dynamic system of uptake and oxidation. In obese Zucker rats, there is increased fatty acid uptake in adipose tissue (30). The observed increase in kinetics corresponded to higher levels of fatty acid transporters, fatty acid translocase and fatty acid transporting protein, suggesting that in the adipose tissue of obese animals, energy storage was more efficient (30,31). Whether chronic intake of a high FR diet leads to altered lipid metabolism, thus contributing to weight gain has yet to be clearly established. The purpose of this study is to examine the effects of FR intake during the suckling period on body weight in adulthood. We also examined aspects of glucose regulation and lipid metabolism.

2. Materials and Methods

2.1. Animals and diet

Pregnant Sprague-Dawley dams (Charles River Laboratories Inc. Wilmington, MA) were housed individually in shoe box cages and had free access to water and standard rat chow (5001 Rodent diet, Canadian Lab Diets, Inc., Leduc, AB). The animal

unit was under 12 hour light/dark cycles and maintained at 22^0 C. Two days after birth, litters were culled to 12 pups/ litter to ensure similar growth of all litters. At 12 days of age, pups from all litters were combined and mixed and then randomly assigned to one of the following suckling diets: suckle controls (SC), rat milk substitute (RMS), high fructose (FR) or high galactose (GAL) formulas. The RMS formula is similar in macro- and micronutrients to rat breast milk. This group served as the control group for our AR process. The high FR milk formula is a RMS-based diet in which 50% of the lactose was substituted with FR. The GAL formula is similar, but substituted with 50% GAL. All diets contained the same amount of total carbohydrate and is isocaloric. Diet composition and macronutrients of the AR diets is shown in Table 1A and 1B. Animals remained on their respective early postnatal diet until 19 days of age, at which time they were weaned to a purified lab chow (AIN 93, Dyets, Bethlehem Pennsylvannia). Composition of this diet is presented in table 1C.

In order to compare the effects of feeding FR in early vs. in later life, from 56-84 days of age, one half of the the SC group was given a purified chow containing 65% FR as the carbohydrate source (AIN 76-base, Dyets, Bethlehem Pennsylvannia) (table 1C). A diagram of the study design is presented in fig. 1. All experimental protocols were approved by the Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee at the University of Alberta, (Edmonton, AB) in accordance with the guidelines of the Canadian Council on Animal Care (Ottawa, Ontario).

2.2. Artificial Rearing of Rat Pups

Rat pups were artificially reared from postnatal days 12 to 19, based on the procedures outlined by Ward et al (32) with minor modifications. Briefly, at 12 days of age, pups were lightly anesthetized (Isofluorane, Abbott Laboratories Limited, Saint-Laurent, Quebec) and fitted with a cheek-tube made from polyethylene tubing (PE10Clay Adams, Parsipanny, NJ) that was held in place by 2 plastic "washers". Cheek tubes were connected to syringes containing 1 of 3 diets described above (Table 1B) on automated syringe pumps (Harvard Apparatus, South Natick, MA. USA). Pumps were set to deliver diet for 12 minutes followed by a 48 minute pause each hour. The volume of diet was calculated to match the growth rates of the SC group. Rats were housed individually in a plastic cup that floated freely in a temperature controlled water bath (38^0 to 40^0 C) for the duration of the suckling period. Two times per day, urination and defecation was gently stimulated by lab personnel using a wet tissue. Body weight was measured daily, and the position of the cheek-tube and general health of each pup was checked frequently throughout this period of the study.

2.3. Regularly Monitored Variables

From the time of weaning onward, body weight and food intake were measured weekly and biweekly. Blood samples were drawn from the tail vein. The plasma was separated by micro-centrifugation (Eppendorf, Westbury NY) and then transferred to a clean tube, and stored frozen at -20^0 C. At a later date, glucose (glucose oxidase , Point Scientific Inc., Lincoln Park, Michigan) and triglyceride (Diagnostic Chemicals Ltd., Charlottetown, PE), concentrations were determined using spectrophotometric methods

(SpectraMax 190, Sunnydale CA) while insulin and leptin concentrations were determined by radioimmunoassay (Linco Research, St. Charles, Missouri). At the end of the study, rats were killed by an overdose of sodium pentabarbital (MTC Pharmaceuticals, Cambridge, Ontario) and the pancreas, liver and retroperitoneal and epididymal fat pads were quickly removed and weighed. Muscle from the hindlimb was excised and used, along with liver and retroperitoneal fat, for assessing long chain fatty acid (LCFA) uptake into giant vesicles (described below).

2.4. Fatty Acid Uptake into Giant Vesicles

Isolation of giant vesicles has been described previously (33). Briefly, thin strips of tissue, placed in Erlenmeyer flasks, were bathed in vesicle preparation medium (140mM KCl/10mM MOPS (pH 7.4)) that had aprotinin (10mg/mL) and collagenase type 2 (150 U/mL) added. Collagenase type 2a was used for adipose while type 2 was used for liver and muscle tissues. The flasks were kept in a shaking water-bath for 1 hour at 34^0C. Tissues were then washed with KCl/MOPS and 10mM EDTA and the supernatant was collected. Percoll® (16% v/v) and aprotinin (0.01 g/mL) was added to the supernatant. The collected supernatant was placed at the bottom of a density gradient made with 4%Nycodenz (w/v) and KCl/MOPS solution and centrifuged at 60xG for 45 min at room temperature. Vesicles, suspended at the interface between the upper and middle layers of the density gradient were harvested and diluted in KCl/MOPS and then centrifuged at 12,000xg for 5 min. KCl/MOPS containing 0.1% w/v BSA was added to the tubes to re-suspend the vesicles. Aliquots of giant vesicles were then used to determine LCFA uptake and protein content.

Fatty acid uptake into giant vescicles was carried out at room temperature by measuring H^3 palmitate (0.3 μCi) and C^{14} mannitol (0.06 μCi) uptake for 15 sec. This process was terminated by adding 1.4 mL of ice-cold KCl/MOPS and 0.1% w/v BSA. The mixture was immediate centrifuged at maximal speed in an Eppendorf micro-centrifuge (Eppendorf, Westbury NY) for 2 min. The supernatant was aspirated and the radioactivity in the precipitate was quantified. Non-specific uptake was measured by adding the 'stop solution' to the vesicles before the addition of the radioactive palmitate solution.

2.5. Statistical Analysis

All results are presented as least squares means ± SEM. Data was analyzed using SAS Systems v.8 (SAS Institutes Inc., Cary, NC). For the variables measured regularly throughout the study, a two way analysis of variance, with time as the repeated factor, was used to test differences among dietary treatments. Statistical significance was accepted at $P<0.05$. Treatment comparisons were carried out by least squares, using the PDIFF option in SAS. For variables measured only at the end of the study, a one-way ANOVA was used to assess differences among diet groups.

3. Results

Regularly Monitored Variables: Body weight – Body weight of pups during the suckling/AR period is shown in fig. 2A. On day 12 (first day of AR), there were no differences in body weight between groups. All artificially reared groups (FR, RMS and GAL) grew in a similar way and there were no differences in body weight during the suckling period. In comparison, the SC pups grew faster than FR and GAL-fed pups on

days 14, 15, 17 and 18 ($p<0.05$). On day 19, the SC pups were heavier compared to all other groups ($p\leq0.001$). The differences in body weight between the SC and artificially reared groups were due to technical difficulties relating to pump operation.

Body weight from weaning to 11 weeks is depicted in fig. 2B. There were no differences in weight from 3 to 7 weeks of age. However, between weeks 8 to 11, the FR group weighed more than SC and FFSc groups and the difference reached statistical significance at weeks 8, 10 and 11 ($p<0.05$). Throughout the study, the RMS and GAL groups had similar body weights to each other and they were not different from the other groups.

Food intake. Food intake was not significantly different among diet treatments at any point during the post-weaning period (fig. 2C). Between weeks 4 to 8, food intake increased in all groups; this reached a plateau and remained at approximately 30 g/day for the remainder of the study. In order to determine whether feed efficiency was different between dietary treatments, daily body weight was calculated as a function of food intake. There were no significant differences throughout the study (fig. 2D).

Circulating serum parameters: Glucose, insulin, leptin & triglyceride - Serum glucose concentrations did not differ among the diet groups throughout the study except at week 11. At this time, glucose concentrations were higher in the FFSc group vs. all other groups ($p<0.05$) (table 2).

Although the FR-fed rats generally had higher circulating serum insulin concentrations compared to all other groups, this did not reach statistical significance due

to large within-group variability. However, statistical significance was noted between the FR and FFsc groups at weeks 8 and 10 ($p<0.05$ at both time points) (table 2).

The FR-fed rats had higher circulating leptin levels compared to all other groups. Significant differences were observed at weeks 8 and 10 ($p<0.05$). Although there was a trend toward higher triglyceride levels in early and late FR-fed rats, this did not reach statistical significance.

Organ weights. Liver weights did not vary between groups (table 3). The mean pancreas weight of the FR group was smaller than the RMS and FFSc groups (1.4 ± 0.13, 1.8 ± 0.12, 1.8 ± 0.13 g, respectively) ($p<0.05$). Epidydimal fat pad of rats in the FR group was heavier than the rats in the FFSc group (12.44 ± 1.72 vs. 7.46 ± 0.71 g) ($p<0.01$). There were no differences in organ weights among other diet groups.

Fatty Acid Uptake into Giant Vesicles. Palmitate uptake into vesicles made from adipose and liver tissue did not differ between groups (figs. 3B and 3C). In skeletal muscle, fatty acid uptake was similar between the RMS, SC, GAL and FFsc groups, however, the FR-fed rats had elevated LCFA uptake (fig. 3A). Statistical significance was noted between the FR and SC groups ($p<0.05$).

4. Discussion

In our study, introduction of FR during the suckling period lead to higher body weight at 11 weeks of age compared to suckle controls. The RMS and GAL groups had similar body weights to the SC rats throughout the study, suggesting that FR ingestion during the suckling period programmed metabolism such that body weight was promoted

in adulthood. The FR-rats also experienced higher circulating insulin, despite normal glycemia and increased leptin levels. Furthermore, fatty acid uptake into skeletal muscle was higher in this group compared to suckled controls. Together, this suggests that the suckling period is malleable to dietary manipulations; in this case, FR intake during this time of life has a persistent effect on growth.

The differences in adult body weight could reflect differences in food intake or energy expenditure among the diet groups. However, there was no significant differences in food intake among the dietary groups throughout the study. Furthermore, the amount of food intake per body weight was not different between groups, indicating that for a given body size, animals from each group were eating the same amount of chow. If the FR rats were eating more per body weight, then we can deduce that intake mechanisms were affected by early FR ingestion, leading to larger weight gain. Our data indicate differences in energy storage, perhaps reflecting lower energy expenditure in the FR group.

In the literature, the effect of FR intake on body weight has been controversial. Hallfrisch et al. (34) found that rats fed a high fat diet (40% of total energy) containing sucrose (30% of total energy) for 8 weeks had higher body weights compared to those that consumed a high fat diet with 30 % of energy from starch (475 ± 7.0 vs. 444 ± 5.9 g) ($p \leq 0.005$). Sucrose-fed rats had larger total epididymal fat mass (12.8 ± 0.67 vs. 10.0 ± 0.47 g) and perirenal fat mass (18.5 ± 0.87 vs. 13.8 ± 0.65 g) ($p<0.005$ for both) in comparison to starch-fed rats. Even after correcting for differences in body weight, both fatness measures were still higher in the sucrose-rats ($p \leq 0.01$ for both). In a 2 week study, Kasim-Karakas et al. (35) demonstrated that hamsters fed a high FR diet became

obese compared to those maintained on a high sucrose feeding or a cornstarch-based control diet. They noted that the FR-fed rats consumed more food compared to the other two groups (26.5 ± 2.8, 11.1 ± 0.6, 8.3 ± 0.8 g/day; FR, sucrose and control). Consequently, these hamsters weighed more than the control or the sucrose group (155± 5, 131 ± 7, 136 ± 7 g, respectively) ($p<0.05$). Similar results were reported by Jurgens et al. (36) whereby, they observed that mice that were allowed ad libitum access to laboratory chow along with a drinking solution that contained 15% of energy in the form of FR dissolved in water for 10 weeks gained more weight compared to those that drank a 10% sucrose solution or those that drank water (47.9 ± 1.4, 44.0 ± 0.7,44.0 ± 1.5 g, respectively) ($p<0.05$). Body composition, determined by nuclear magnetic resonance, revealed that the FR-mice had higher body fat content (10.5 ± 2.5, 7.9 ± 1.0, 5.4 ± 1.5%; FR, sucrose, water) ($p< 0.05$). Cumulative energy intake of chow was lower in the FR group, whereas, both, the sucrose and water groups had higher intakes (836.4 ± 28.6, 918.0 ±27.7, 990.5 ± 36.7 kcal, respectively) ($p<0.01$ FR vs. water). Therefore, other mechanisms beside food intake promoted the increased weight gain and adiposity in the FR-mice.

In contrast, other investigators have observed lower amounts of weight gain with FR feeding. In a study to test the effect of different diets on eating patterns, 344 rats at the age of 3 months were assigned to either a standard rat chow or high FR diet for 10 days (37). The researchers found that the rats on the FR diet ate less compared to the controls (16.8 ± 0.9 vs.19.9 ± 0.7 g/day) ($p\leq0.001$). As a result, the FR- fed rats had smaller gains in bodyweight compared to control rats. Similar results were reported for a

12 week study where 24 day old rats were fed either a FR-enriched or starch diet (38). The FR-fed rats grew more slowly than those fed the starch diet. At the end of the 12 weeks, the FR group gained 94 g, while the starch group gained 113 g ($p<0.05$). It should be noted that the authors presented median weights, rather than the usual mean weights, therefore, SEM were not available. From these investigations, the effect of dietary FR on body weight has yet to be established.

Our study showed that introduction of high FR during the suckling period lead to increased body weight in later life compared to rats that consumed mother's milk, a rat milk substitute, or rats whose formulae contained galactose. Although absolute epididymal fat pad weight was significantly higher in the early FR-fed rats compared to the suckle controls fed FR late in life, when corrected for differences in body weight, there were no differences between dietary treatments. Thus, suggesting that perhaps, both lean and fat mass accumulation was higher in FR-rats. Body composition analysis was not part of this study because of tissue requirements for other measures. Therefore at this time it is unclear whether the increased body weight reflects energy deposition in both lean and fat tissue. Future studies that might help elucidate these differences are: 1) carcass analysis using bomb calorimetry to determine body composition and 2) histology work to determine fat particle size and number in these tissues.

In the FR-fed rats, palmitate uptake into giant vesicles made from hindlimb skeletal muscle was significantly higher compared to uptake in muscle of SC rats ($p<0.05$). There were no differences in LCFA uptake into vesicles made from adipose or liver tissues among treatment groups. Utilizing obese Zucker rats as a model of altered lipid metabolism, Luiken et al (39) observed that in both, hindlimb muscle and adipose

tissue there was a 1.8 fold increase ($p<0.05$ for both) in vesicular uptake compared to lean littermates. To determine whether obese and type 2 diabetic individuals have different lipid metabolism mechanisms compared to lean human subjects, Bonen et al (40) performed uptake studies utilizing vesicles made from rectus abdominus muscle. They observed that in comparison to lean subjects, people who were obese and those with diabetes had significantly higher palmitate uptake (3.8 fold and 4.3 fold, respectively vs. lean subjects) ($p<0.05$). To elucidate whether the increased fatty acid uptake was associated with lipid accumulation in muscle, the authors measured the rate of palmitate esterification and intramuscular triacylglycerol (TG) content. In the muscle of obese subjects, the rate of palmitate esterification into TG was 3-fold higher compared to muscle of lean subjects ($p<0.05$). Unfortunately, data in overweight and type 2 diabetics was not presented. Intramuscular TG content was also higher in obese (2-fold) and diabetics (3-fold) compared to lean subjects ($p<0.05$ for both). Therefore, these studies suggest that in obese and insulin resistant states, higher LCFA transport into skeletal muscle may contribute to increased esterfication into fatty deposits within muscle fibre. In our study, the increased LCFA uptake observed in the muscle of adult rats fed FR in early life suggests long-term perturbed lipid metabolism.

Many investigators have shown that short term feeding of a high FR diet to rodents results in hyperinsulinemia, glucose intolerance and insulin resistance (25, 26, 27, 28,41). Insulin is one key hormone regulating whole body energy balance (reviewed in 42). The present study utilizes the concept of metabolic programming to examine the long-term consequences of early introduction of dietary FR. We observed that serum insulin concentrations in the FR group was higher compared to all other groups between

weeks 6 to 11 and the differences reached statistical significance at 8 and 10 weeks of age (table 2).

Despite the hyperinsulinmenia observed in animals fed FR during the suckling period, glucose concentrations of this group did not differ from the other diet groups. The rats in the FFSc group had higher serum glucose levels at week 11 ($p<0.05$) without a concomitant increase in serum insulin. This may indicate that in these older rats, ingestion of a high FR diet lead to altered glucose homeostasis without an observed change in serum insulin, while ingestion of FR in the pre-weaning stage lead to alterations in insulin secretion. It suggests that the effects of dietary FR on metabolism may vary across the lifespan.

In adult life, absolute circulating leptin levels in the FR-rats were higher compared to all other dietary groups at weeks 8 and 10 ($p<0.05$) . When corrected for differences in bodyweight, early FR-fed rats had higher leptin levels compared to suckle controls and suckle controls fed FR in late life ($p<0.05$) 0.05; FR vs.Sc and FFSc at weeks 8, 10, 11) (table 2), thus indicating possible leptin resistance.

The observation of hyperinsulinemia but similar glucose concentrations suggests that early exposure to FR lead to persistent insulin resistance that manifests as the animals age and/or are post-pubertal. Although the mechanism of insulin resistance has yet to be established, muscular insulin resistance can result in re-distribution of glucose utilization to adipocytes, leading to larger fat mass (43). Using the muscle specific insulin receptor knockout mice (MIRKO) model, Kim et al (44) determined insulin-stimulated glucose transport in the muscle of mice undergoing hyperinsulinemic-euglycemic clamps by injection of radioactive 2-deoxyglucose. They observed that in the

skeletal muscle of MIRKO mice, insulin-stimulated glucose transport activity was decreased compared to controls (57 ± 11 vs. 219 ± 23 nmol/g/min) ($p \leq 0.005$). In contrast, epididymal white adipose tissue of MIRKO mice showed 3-fold increase in glucose uptake (26 ± 4 vs. 9 ± nmol/g/min in controls) ($p \leq 0.005$).

Other researchers used the transgenic mouse model of *in vivo* insulin resistance to demonstrate the dynamic relationship of insulin-stimulated glucose utilization between muscle and adipose tissues. In these mice, overexpression of kinase-deficient human insulin receptor in muscle results in impairment of insulin-stimulated muscle receptor activity (45). Utilizing hindlimb insulin perfusions with 3-0-methyl-D-glucose, Moller et al (46) demonstrated that insulin stimulated glucose uptake was significantly reduced by 30% in soleus muscle and 40% in gastrocnemius muscle of transgenic mice in comparison to controls ($p \leq 0.01$). Body composition analysis revealed that transgenic mice had 38% higher body lipid content than control mice ($p \leq 0.005$). These studies illustrate that muscular insulin resistance can lead to re-allocation of glucose metabolism to adipocytes, and as such, promoted higher body lipid content. Our early FR-fed rats had higher LCFA uptake into muscle tissue and exhibited hyperinsulinemia in comparison to SC and FFSc. In addition, the heavier fat pad observed in these rats compared to suckled rats fed a high FR diet in later life does not exclude the possibility of programmed re-routing of substrate utilization to adipocytes.

In summary, early introduction of FR resulted in increased LCFA uptake into skeletal muscle, higher circulating insulin and leptin levels. These rats had larger body weight in adult life, potentially due to altered fuel utilization via derangements in insulin and leptin control of peripheral storage. Currently, the programming period for increased

adult body weight and adiposity has yet to be defined. The postnatal period is one that can be malleable to manipulations in nutritional intake. As such, physiological changes incurred during suckling due to high FR ingestion may result in lifelong changes in cellular function. Additional studies are needed to investigate molecular pathways, thus shedding insight into mechanisms contributing to observed changes in biological markers.

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6. Tables and figures for chapter 2

Table 1A. Composition of milk formulas used in the artificial rearing process

Ingredients	RMS formula	High FR formula	High GAL formula
	Amount *		
Casein	48.7g	48.7g	48.7g
Whey protein	73.1g	73.1g	73.1g
Purified water	786.4ml	786.4ml	786.4ml
1% Lecithin	0.01g	0.01g	0.01g
Non-calcium mineral mixture[a]	5.5g	5.5g	5.5g
CuSO4 solution (0.031g/ml)	0.9ml	0.9ml	0.9ml
ZnSO4 solution (0.38g/ml)	0.3ml	0.3ml	0.3ml
Caritine (0.1g/ml)	0.4ml	0.4ml	0.4ml
Creatine (0.01g/ml)	6.4ml	6.4ml	6.4ml
Ethanolamine	0.03ml	0.03ml	0.03ml
Ca(OH)2	1.4g	1.4g	1.4g
CaHPO4	7.3g	7.3g	7.3g
CaCl2.2H2O	1.8g	1.8g	1.8g
Custom amino acid mixture[b]	0.9g	0.9g	0.9g
Tecklad Vitamin mixture	3.6g	3.6g	3.6g
Custom Vitamin diet fortification mix[c]	0.5g	0.5g	0.5g
Lactose	29.5g	14.8g	14.8g
Fructose	0.0g	14.8g	0.0g
Galatose	0.0g	0.0g	14.8g
Fat mixture[d]	127.3g	127.3g	127.3g

* Total volume of each type of formulae is 1 liter.

[a] KH2PO4 (210.5g/250g), MgSO4 (38g/250g), FeSO4.7H2) (1g/250g), KI (0.0725g/250g), NaF (0.0615g/250g), AlSO4 (0.0390g/250g)MnSO4 (0.0105g/250g)

[b] arginine (51g/100g), glycine (31g/100g), taurine (16g/100g), picolinic acid (2g/100g)

[c] riboflavin (1.7g/100g), niacin (2.7g/100g), pyridoxal (1.4g/100g), inositol (94.2/100g)

[d] medium chain triglyceride oil (35g/140g), coconut oil (67.2g/140g), soy (23.8g/140g) olive oil (14g/140g)

Table 1B. Macronutrient compositions and caloric distribution of rat milk and milk formulae used in the artificial rearing process

Nutrient	Rat milk*	RMS	FR	GAL
	g/100 mL of formula			
Protein	9.2 (23%)**	12.2 (28%)	12.2 (28%)	12.2 (28%)
Carbohydrate:				
*Lactose****	3.0 (6%)	3.0 (6%)	1.5 (3%)	1.5 (3%)
Fructose	-	-	1.5 (3%)	-
Galatose	1.5 (3%)	1.5 (3%)	0.8 (1.5%)	2.3 (4.5%)
Glucose	1.5 (3%)	1.5 (3%)	0.8 (1.5%)	0.8 (1.5%)
Fat	12.3 (70%)	12.7 (66%)	12.7 (66%)	12.7 (66%)

* rat milk composition as reported by Dymsza HA et al (ref #15)

** % calories in brackets

*** lactose is comprised of ½ glucose & ½ galactose

Table 1C. Composition of the purified rat diet (AIN 93) and purified rat chow with 65% FR as the source of carbohydrate (AIN 76)*

Ingredients	Purified rat diet	High FR diet
	g/100g	
Casein	20.0g	20.0g
Cornstarch	39.7g	0.0g
Dyetrose	13.2g	0.0g
Sucrose	10.0g	0.0g
Fructose	0.0g	65.0g
Cellulose	5.0g	2.5g
Soybean oil	7.0g	7.0g
t-Butylhydroquinone	0.0001g	0.0001g
Salt mix[a]	3.5g	3.5g
Vitamin mix[b]	1.0g	1.0g
L-Cystine	0.3g	0.3g
Choline bitartrate	0.3g	0.3g

*Dyets, Bethlehem Pennsylvannia

[a] $CaC0_3$ (35.7g/100g), $KHP0_4$ (19.6g/100g), K_3C_6H507 (7.0g/100g), NaCl (7.4g/100g), K_2S04 (4.6g/100g), Mg0 (2.4g/100g), FeC_6H_807 (0.6g/100g), ZnCO3 (0.2g/100g), MnC03 (0.01g/100g), CuC03 (0.003g/100g), KI03 (0.0001g/100g), NaSe04 (0.0001g/100g), $NH_46Mo7024.4H20$ (0.00008g/100g), $Na_2Si03.9H20$ (0.1g/100g), CrK(S04)2.12H2) (0.003g/100g)

[b] nicacin (3g/1000g), pantothenate (1.6g/1000g), pyridoxine (0.7g/1000g), thiamine (0.6g/1000g), riboflavin (0.6g/1000g), folic acid (0.2g/1000g), biotin (0.02g/1000g), vit. E (15g/1000g), vit. B12 (2.5g/1000g), vit. A (0.8g/1000g), vit. D (0.25g/1000g), vit K (7.5g/1000g)

Table 2. Plasma glucose, insulin, triglyceride and leptin concentrations at weeks 8,10 and 11

Parameters	Diet	wk 8	wk 10	wk 11	P value
glucose (mM)	Fr	7.5 ± 0.4	7.1 ± 0.3	9.0 ± 0.3 *	* $p<0.05$ (FR vs. Gal)
	Sc	7.6 ± 0.4	7.2 ± 0.4	8.7 ± 0.3	† $p<0.05$ (FFSc vs. all others
	RMS	7.2 ± 0.3	6.7 ± 0.2	8.9 ± 0.2	
	Gal	7.3 ± 0.4	6.9 ± 0.3	8.0 ± 0.3	
	FFSc	6.9 ± 0.3	7.1 ± 0.3	9.8 ± 0.3 †	
insulin (ng/mL)	Fr	0.52 ± 0.10 *	0.59 ± 0.12 *	0.63 ± 0.10	* $p<0.05$ (FR vs. FFSc)
	Sc	0.26 ± 0.14	0.27 ± 0.17	0.51 ± 0.15	
	RMS	0.46 ± 0.08	0.38 ± 0.10	0.44 ± 0.08	
	Gal	0.27 ± 0.10	0.38 ± 0.12	0.37 ± 0.10	
	FFSc	0.17 ± 0.09	0.18 ± 0.11	0.38 ± 0.09	
triglyceride (mg/dL)	Fr	129 ± 16	118 ± 19	219 ± 33	
	Sc	118 ± 16	103 ± 19	163 ± 33	
	RMS	125 ± 16	111 ± 19	161 ± 33	
	Gal	92 ± 18	109 ± 21	144 ± 37	
	FFSc	100 ± 18	141 ± 21	223 ± 37	

Data are mean ± SEM.

Table 2 continued. Plasma glucose, insulin, triglyceride and leptin concentrations at weeks 8,10 and 11

Parameters	Diet	wk 8	wk 10	wk 11	P value
leptin (ng/mL)	Fr	5.4 ± 0.6 *	11.3 ± 1.6†	7.7 ± 1.3	* p<0.05 (FR vs. Sc, RMS, FFSc)
	Sc	2.8 ± 0.7	4.5 ± 2.0	4.7 ± 1.6	† p<0.05 (FR vs. all others)
	RMS	3.5 ± 0.6	6.3 ± 1.6	5.9 ± 1.3	
	Gal	3.7 ± 0.6	6.1 ± 1.7	4.7 ± 1.4	
	FFSc	3.0 ± 0.7	3.1 ± 2.0	3.5 ± 1.6	
% leptin (ng/g fat pad) *100	Fr	-	-	66.2 ± 9.1	
	Sc	-	-	59.3 ± 9.1	
	RMS	-	-	63.6 ± 8.3	
	Gal	-	-	55.4 ± 9.1	
	FFSc	-	-	42.0 ± 9.1	

Data are mean ± SEM.

Table 3. Liver, pancreas and epidydimal fat pad weight of animals in the four different dietary treatments

Diet	Liver	Pancreas	Epidydimal fat pad
	absolute weight (g) *g/100g bodyweight*		
FR	24.8 ± 1.7 *4.0 ± 0.2*	1.4 ± 0.1 * *0.2 ± 0.0*	12.4 ± 1.2 † *2.0 ± 0.1*
RMS	21.0 ± 1.6 *3.8 ± 0.2*	1.8 ± 0.1 *0.3 ± 0.0*	9.3 ± 1.1 *1.7 ± 0.1*
GAL	19.5 ± 1.7 *3.5 ± 0.2*	1.5 ± 0.1 *0.3 ± 0.0*	9.1 ± 1.2 *1.6 ± 0.1*
SC	20.0 ± 1.9 *3.8 ± 0.2*	1.6 ± 0.1 *0.3 ± 0.0*	9.2 ± 1.2 *1.8 ± 0.1*
FFSc	22.4 ± 1.7 *4.5 ± 0.2*	1.8 ± 0.1 *0.4 ± 0.0*	7.5 ± 1.2 *1.5 ± 0.1*

Data are mean ± SEM.

* $p<0.05$ FR vs. FFSc and RMS, † $p<0.01$ between FR and FFSc

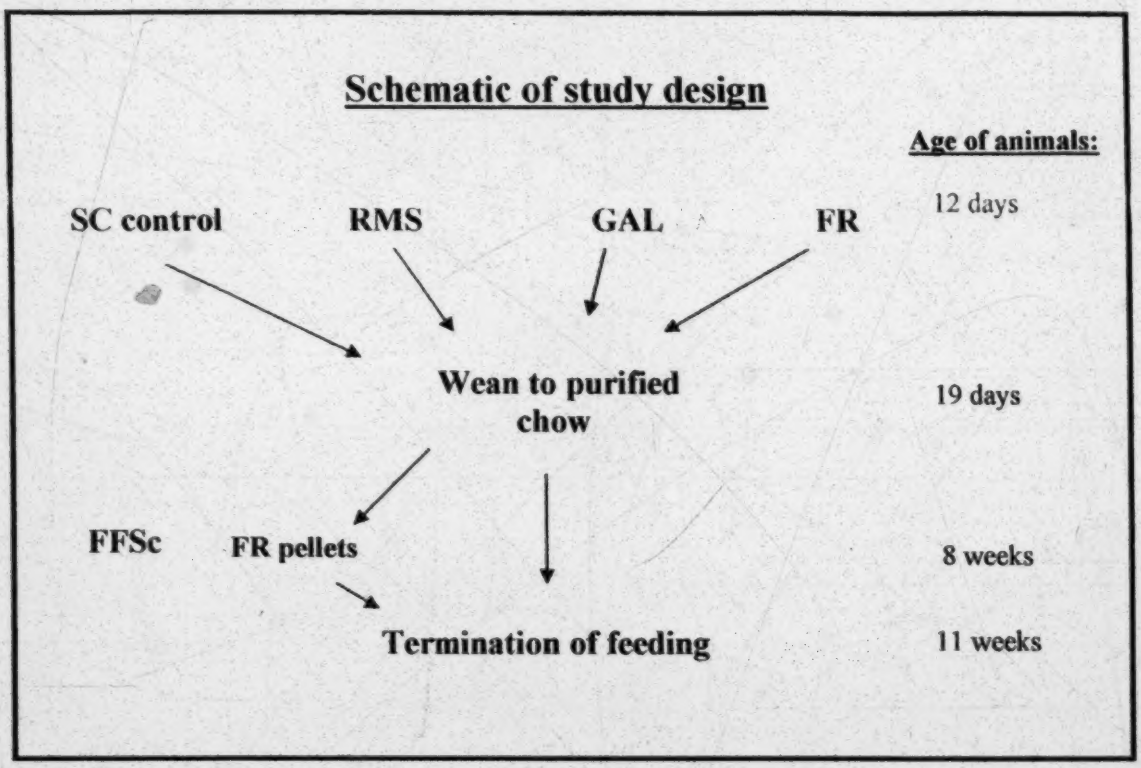

Fig. 1. Flow diagram of the study design. We used the artificial rearing method to introduce the respective milk formulae to the pups at 12 days of age. They were kept on these treatments until 19 days of age, at which point, they were weaned to a purified rat chow. All rats were maintained on this diet until 11 weeks. In order to examine the effects of high fructose intakes in later life, we removed half the animals (n=5) from the suckle control group that were eating the purified rat chow and changed their feed to a high FR diet (65% of total calories) at 8 weeks of age (the FFSc group).

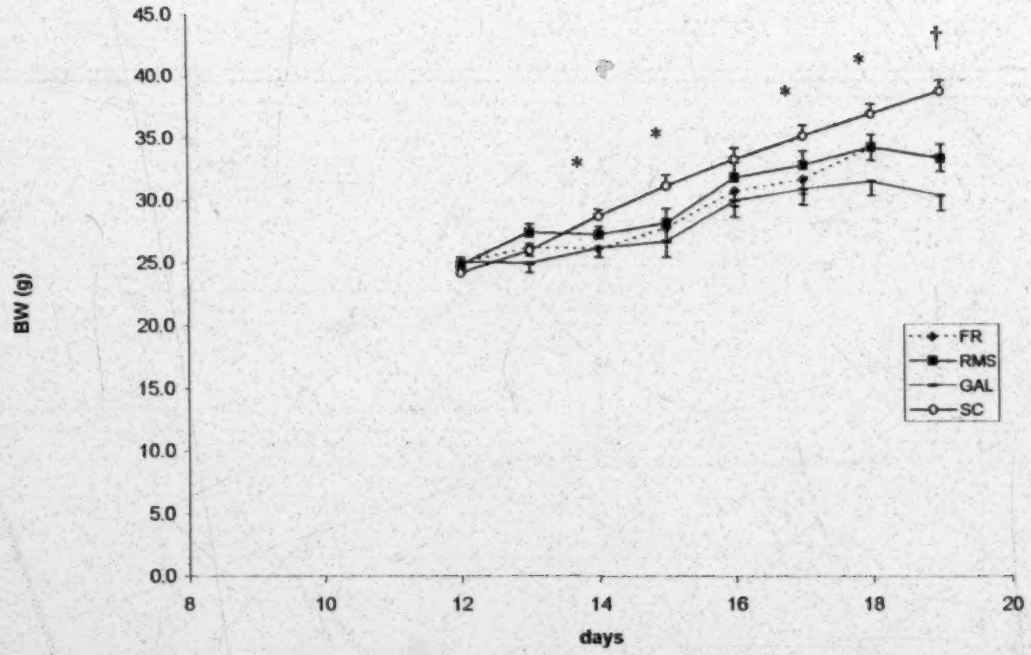

Fig. 2A. Body weight of pups during the artificial rearing (FR, RMS & GAL groups) or suckling period (SC group) from days 12 to 19 postnatal; expressed as mean ± SEM; * $p<0.05$ SC vs. FR and GAL, † $p<0.001$ SC vs all others.

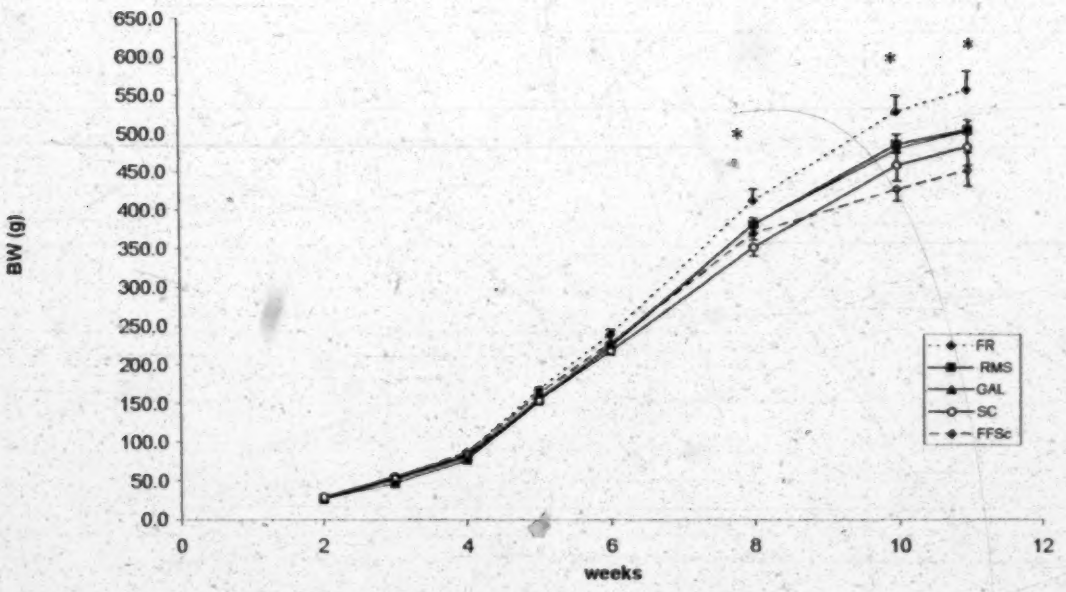

Fig. 2B. Body weight of rats in the different dietary treatments from 2 to 11 weeks of age. At 2 weeks of age, the pups were either suckling on the dam or artificially reared. All pups were weaned at 19 days of age onto a purified rat chow. The rats in the FR, RMS, GAL and SC groups were maintained on this diet until they were 11 weeks of age. Rats in the FFSc group were fed the purified rat chow until 8 weeks of age, after that, they were switched to a purified rat chow containing 65% FR as the source of carbohydrate; expressed as mean ± SEM; * $p<0.05$ FR vs. FFSc and SC.

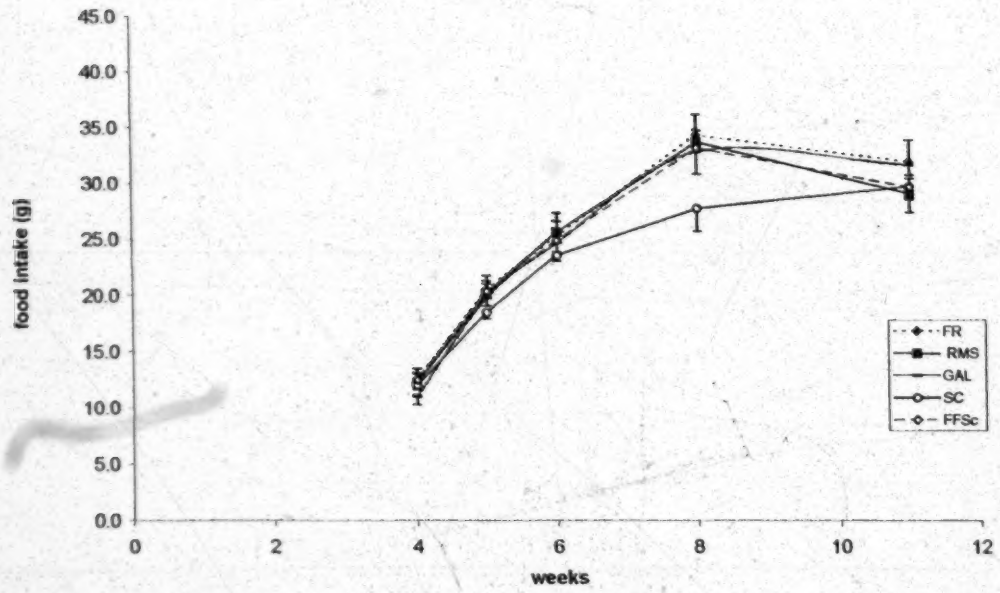

Fig. 2C. Food intake of rats in the different dietary groups during the post-weaning period; expressed as mean ± SEM.

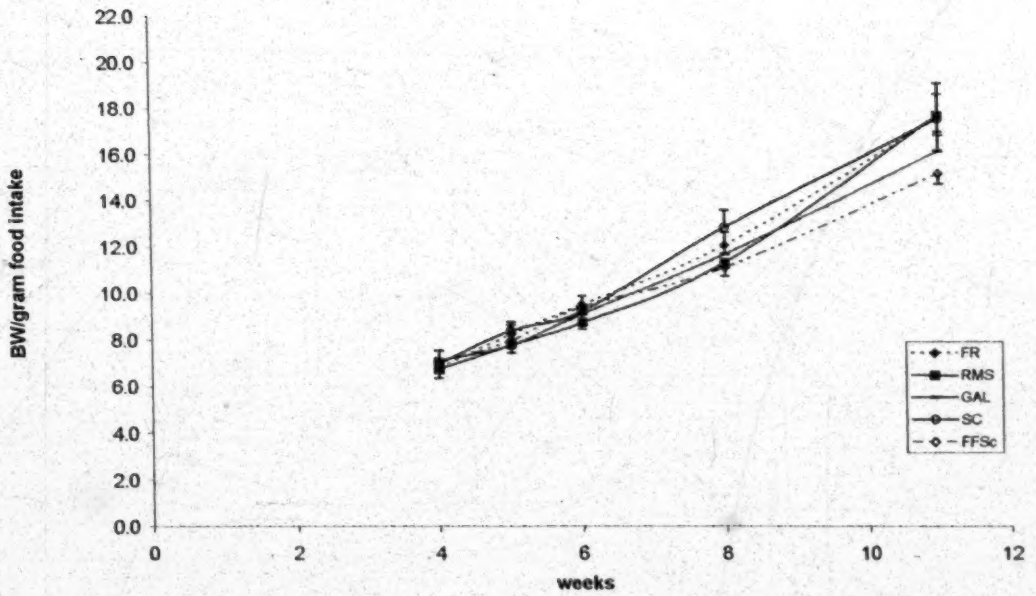

Fig. 2D. The feed efficiency of rats in each diet group, calculated by dividing weight gain by daily food intake; expressed as mean ± SEM.

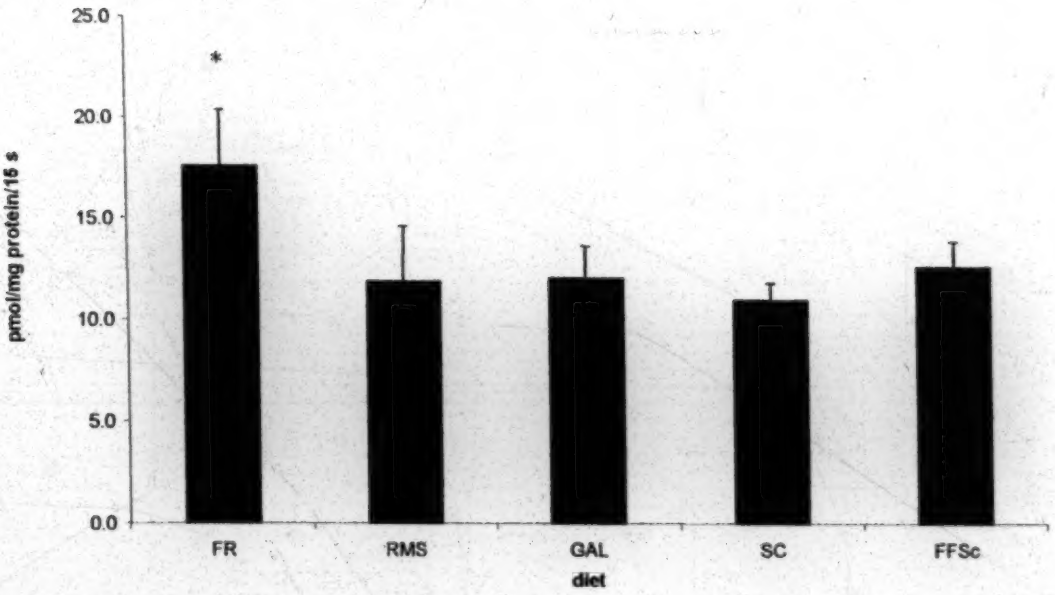

Fig. 3A. The rate of palmitate uptake into vesicles made from skeletal muscle; expressed as mean ± SEM; * $p<0.05$ FR vs. SC.

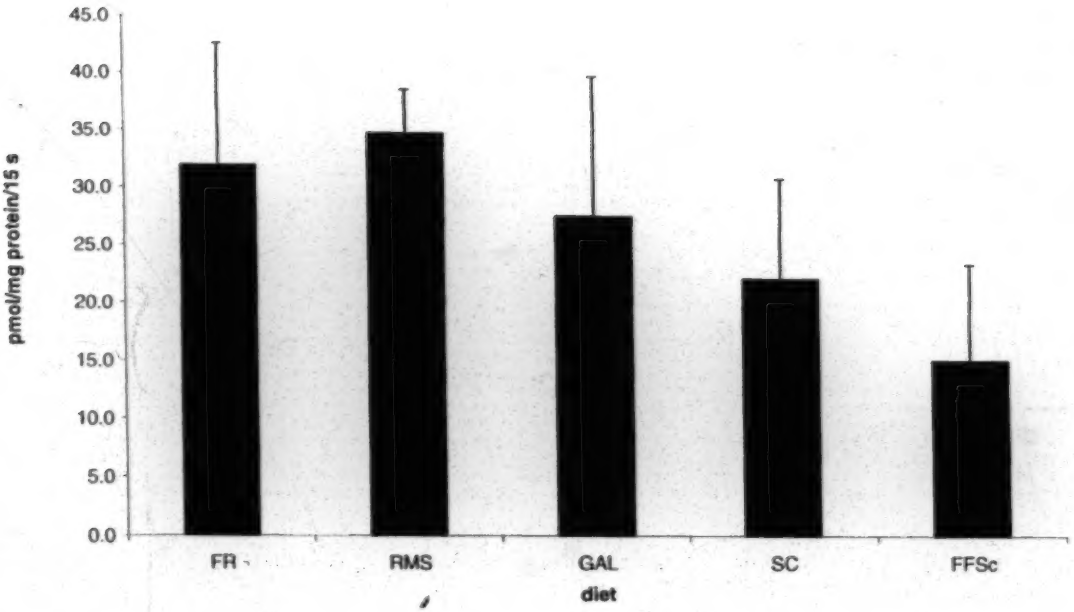

Fig. 3B. The rate of palmitate uptake into vesicles made from liver tissue; expressed as mean ± SEM; expressed in mean ± SEM.

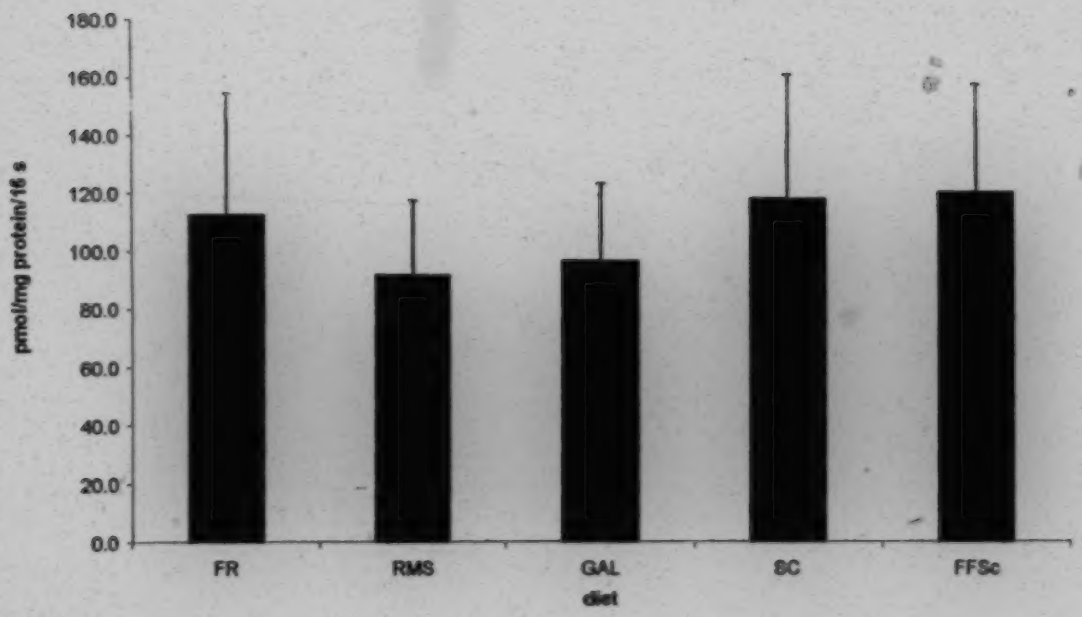

Fig. 3C. The rate of palmitate uptake into vesicles made from adipose tissue; expressed as mean ± SEM.

Chapter 3: Results from cohorts #2 and #3

1. Introduction

Obesity is currently one of the high-ranking health issues plaguing developed nations. Co-morbidities associated with excessive weight include insulin resistance, diabetes, hypertension, coronary heart diseases and possibly some cancers. In the US, approximately 2 out of every 3 adults are overweight and 1 out of 3 is obese (1). Canadians fare better, however, the statistics are comparable with 1 out of 2 adults classified as overweight and approximately 1 out of 5 are in the obese category (2).

These problems are also present in children and adolescents. The prevalence of overweight and obesity in Canadian children has increased over the past several decades. Although there are slight discrepancies regarding how to best classify excessive weight in children, it is clear that the trend is upward (3). Between 1981 and 1996, the percentage of overweight boys aged 7 – 13 years rose from 11 % to 33%, while overweight in girls increased from 13% to 27%. The obesity rate for this group of children changed from 2% to 10% in boys and 2% to 9% in girls (4). This is alarming because obesity in childhood, and particularly in adolescence, is a key predictor of obesity in adulthood (5).

Although many factors play a role in energy balance, diet represents the intake side of the energy homeostasis equation. Increased consumption of low nutrient, but energy dense foods, including caloric sweeteners, may exacerbate the propensity towards

weight gain in a population that is increasingly sedentary (6). Utilizing food disappearance data, Popkin & Nielsen estimated that the US population has increased the use of caloric sweeteners by 22% within the last 4 decades (7). Of this, 80% of the increase are in the form of soft drinks and sugared fruit drinks. Data from the NHANES 1999 – 2000 showed that sweetened foods such as soft drinks, cakes, sweet rolls and pastries make up approximately 23% of total energy intake (8). The two most common sweeteners used in the food industry are high fructose corn syrup (HFCS) and sucrose (9,10). Sucrose is comprised of 50% fructose (FR) and 50% glucose while HFCS contains 42% to 55% FR, depending on the source.

Fructose metabolism differs from glucose in that it does not stimulate acute insulin secretion (11), may affect acute leptin levels (12) and favours lipogenesis (13). Experimental evidence in animals shows that although FR does not stimulate insulin response directly, long-term consumption of high FR leads to hyperinsulinemia (14,15,16,17) and dyslipidemia (18,19). Alterations in endocrine regulation of energy balance as a result of increased FR intake may distort energy homeostasis. Currently, the literature on the effects of FR ingestion on body weight and fat mass in both, animals and humans are conflicting. Some, but not all studies in animals, report a positive energy balance associated with high FR intakes (19,20). Mice that had ad libitum access to standard chow along with a drinking solution containing 15% of energy from dissolved in water for 10 weeks had increased body weight and fat mass compared to those that drank water and consumed chow ($p<0.05$ for both parameters) (21). Similarly, rats maintained on a high fat diet (40% of total energy) supplemented with sucrose (30% of total energy) weighed more and had larger fat mass compared to those fed a high fat diet containing

30% starch ($p< 0.005$) (22). In contrast, adult rats fed a high FR diet (60% of total calories) for 10 days had weight gains that were similar to those fed a high glucose diet (60% of total calories) (23). A longer term study demonstrated that rats fed a high FR diet (60% of total calories) for 12 weeks grew more slowly than those that ate a starch-based diet (60% of total calories) ($p<0.05$) (24).

In humans, there are similar discrepancies in weight outcomes resulting from high FR consumption. Epidemiological & experimental investigations in children and adults suggest that high intakes of FR from liquid sources may be associated with increased food intake and weight gain (25,26,27,28,29,30). In a longitudinal study conducted over a 2 year period in school-aged children, the authors found that these children were 1.6 times more likely to become obese with each additional intake (12 oz) of sugar-sweetened drinks (31). This may be due to a lack of caloric compensation following consumption of soft-drinks (32,33,34). On the contrary, utilizing data from the US Department of Agriculture's Continuing Survey of Food Intake by Individuals 1994 – 1996, Forshee & Storey reported no association between body mass index and consumption of regular carbonated beverages or fruit drinks (35). There are several reasons for the variations in results, including differences in data collection methods and measurement techniques.

Although the evidence to assess both intake and body weight resulting from high FR intakes is inconclusive, it is generally agreed that the temporal rise in childhood obesity coincides with increased intakes of soft drinks (36). Furthermore, children are the largest consumer of sweetened beverages (37). Currently, the association between FR-sweetened beverage consumption in young life on future body mass is unclear. We

utilized the concept of metabolic programming to determine whether high intakes of FR during early development lead to changes in body weight through out the lifespan.

2. Materials and Methods

The methods used in this chapter are the same as those employed in chapter 2, with the exception of oral glucose tolerance tests. Therefore, descriptions in each step of this section are summaries of those described in chapter 2

2.1. Animals and diet

Pregnant Sprague-Dawley dams (Charles River Laboratories Inc. Wilmington, MA) were housed individually in shoe box cages and had free access to water and standard rat chow (5001 Rodent diet, Canadian Lab Diets, Inc., Leduc, AB). Litters were culled at two days after birth to 12 pups/ litter to ensure similar growth of all litters. At 12 days of age, pups from all litters were combined and mixed and then randomly assigned to an early postnatal dietary group. These groups were as follows: Suckle Controls (SC), in which the pups remained with a foster dam. The other pups were assigned to be artificially reared (described below) on 1 of two diets: Rat Milk Substitute (RMS), macro- and micronutrient composition similar to rat breast milk); Fructose (FR), a RMS-based diet in which 50% of the lactose was substituted with FR. All diets contained the same amount of total carbohydrate. Animals were fed their respective early postnatal diet until 19 days of age, at which time they were weaned to a purified lab chow (AIN 93, Dyets, Bethlehem Pennsylvannia). It should be noted that in this chapter, there

were two cohorts of animals. The materials and methods used were the same in both two cohorts.

All experimental protocols were approved by the Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee at the University of Alberta, (Edmonton, AB) in accordance with the guidelines of the Canadian Council on Animal Care (Ottawa, Ontario).

2.2. Artificial Rearing of Rat Pups

Rat pups were artificially reared from postnatal days 12 to 19, based on the procedures outlined by Ward et al (38) with minor modifications. They were fed 1 of the 2 diets described above: RMS and FR. Automatic syringe pumps (Harvard Apparatus, South Natick, MA. USA) were set to deliver diet for 12 minutes followed by a 48 minute pause each hour. The volume of diet was calculated to match the growth rates of the SC group. Bodyweight was measured daily, and the position of the cheek-tube and general health of each pup was checked frequently throughout this period of the study.

2.3. Regularly Monitored Variables

From the time of weaning onward, bodyweight and food intake were measured weekly and biweekly respectively. Blood samples were drawn from the tail vein biweekly, the plasma was separated by micro-centrifugation (Eppendorf, Westbury NY), transferred to a clean tube, and stored frozen at -20 ^{0} C. At a later date, glucose (glucose oxidase , Point Scientific Inc., Lincoln Park, Michigan) and triglyceride concentrations (Diagnostic Chemicals Ltd., Charlottetown PE) were determined using

spectrophotometric methods (SpectraMax 190, Sunnydale CA) while insulin and leptin concentrations were determined by radioimmunoassay (Linco Research, St. Charles, Missouri). At the end of the study, rats were killed by an overdose of sodium pentabarbital (MTC Pharmaceuticals, Cambridge, Ontario) and the pancreas, liver and retroperitoneal and epididymal fat pads were quickly removed and weighed.

2.4. Oral glucose tolerance test (OGTT) at 10 weeks of age

Rats were fasted for 12 hours overnight, during which time they were given unlimited access to water. On the morning of the blood collection, the rats were kept warm in a shoe box under a heating lamp for 10 minutes. Animals were monitored carefully for signs of dehydration and stress. The glucose load was calculated at 3g/kg bodyweight and delivered using an 18 G gavage needle. Blood was collected via a tail vein prick at baseline and every 15, 30, 45, 60 and 90 minutes post gavage and placed into sodium heparin coated tubes (less than 10 units heparin per mL blood). Samples were placed on ice, centrifuged and subsequently plasma was aliquot into clean vials and stored at -20 ^{0} C for further analysis of glucose and insulin and triglyceride.

2.5. Statistical Analysis

All results are presented as least squares means ± SEM. Data was analyzed using SAS Systems v.8 (SAS Institutes Inc., Cary, NC). For the variables measured regularly throughout the study, a two way analysis of variance, with time as the repeated factor, was used to test differences among dietary treatments. Statistical significance was accepted at $P<0.05$. Treatment comparisons were carried out by least squares, using the

PDIFF option in SAS. For variables measured only at the end of the study, a one-way ANOVA was used to assess differences among diet groups.

3. Results

Regularly Monitored Variables: Body weight – All pups grew steadily during the AR/suckling period (fig.1A). There were no differences in body weight at the beginning of the AR period (day 12). On subsequent days, the pups fed FR diet weighed more than the pups fed the RMS formula on days 13, 14, 15, 16 and 18 postnatal ($p<0.05$). SC pups were heavier than RMS pups on days 14 and 15 ($p<0.05$). In comparison to the growth of SC pups, FR pups grew in a similar manner.

Body weight of animals in the post-weaning period did not differ among treatment groups (fig.1B). In comparison with the rats in cohort #1 (reported in Chapter 2), the RMS group in this cohort attained similar weights at 11 weeks of age (505.3 ± 20.3 vs. 497.3 ± 10.1 g, cohort 1 vs. 2). The SC group in cohort # 1 weighed approximately 15 g more compared to the rats raised in this cohort (482.8 ± 22.2 vs 466.9 ± 12.3 g, cohort 1 vs. 2). The largest difference (approximately 87 g) was observed in the FR group, whereby, FR rats in cohort #1 weighed more than the FR rats reported in this chapter (556.9 ± 22.2 vs. 470.2 ± 10.1 g, cohort 1 vs. 2). The overall growth pattern was similar between the two cohorts whereby animals grew rapidly from weeks 4 to 8 and then tapered off until week 11.

Food intake. As was observed in cohort 2, food intake was not significantly different among diet treatments in the post-weaning period (fig.2). The intake trend was also similar between the two cohorts. In the early weeks post-weaning, food intake

increased up to week 8, then plateau and remained at approximately 30 g/day until week 11.

Non-fasting plasma: Glucose, insulin, leptin & triglyceride concentrations - Serum glucose levels did not differ among the diet groups at 6 and 8 weeks of age. At week 11, the FR group had higher circulating glucose concentration compared to the SC group ($p<0.05$) (table 1). Circulating serum insulin and leptin concentrations did not differ among diet groups throughout the study. At weeks 8 & 11, the RMS group tended to have higher non-fasting insulin concentrations compared to both FR and SC groups; however, due to large within-group variability, this difference did not reach statistical significance. Triglyceride levels were not different between among the treatment groups at any time during the study.

Fasting plasma: Glucose, insulin & triglyceride – At 10 weeks of age, an OGTT was performed on overnight fasted rats. We determined the plasma glucose, insulin and triglyceride concentrations of the fasting (baseline) samples (table 2). Although we obtained values with a smaller margin of variability, we did not observe differences between the treatment groups.

OGTT parameters: Glucose & insulin - Glucose concentrations were higher in the RMS group compared to the FR group at 15, 60 and 90 min post gavage ($p<0.05$) (fig. 3A). At 15 min, the RMS rats also had higher glucose levels compared to the SC rats ($p<0.05$), but from 30 min onward, glucose levels did not differ between rats from the RMS and SC

groups. Insulin response to an oral glucose challenge was similar between the RMS and FR groups. Both of these diet treatments tended to be higher compared to the SC group (fig 3B); however, there was a significant treatment*time interaction ($p<0.001$). The graph of this interaction (fig 3C) reveals that both, the RMS and FR groups had significantly higher insulin concentrations compared to the SC group at all time points except 45 min ($p<0.05$ for all time points mentioned).

Organ weights. The liver from the RMS rats was heavier compared to SC rats, both in absolute weight and when expressed as a percentage of total bodyweight ($p<0.05$ for both) (table 3). Body fatness, as measured by epidydimal fat pad weight was not different among diet groups.

4. Discussion

The main objective of this investigation was to determine whether early FR intake leads to increased body weight in adulthood. We did not observe differences in body weight among the dietary treatments. Food intake was also similar in all treatment groups, indicating that intake was not affected by early diet manipulation. In terms of metabolic profile, non-fasting insulin, triglyceride and leptin concentrations were similar among all groups throughout the study. Circulating glucose concentrations were similar in all groups at weeks 6 and 8. Although, at week 11, the FR-fed rats had higher concentrations compared to the SC rats ($p\leq0.05$). Fasting plasma triglyceride concentrations were not different among the diet groups. Thus, the data show that energy balance and metabolic profile was not affected by early FR-feeding in this cohort of rats.

During the oral glucose tolerance tests, the RMS group tended to have higher glucose levels at fasting and at 90 min post gavage compared to the other two treatment groups. A statistically significant difference was noted at 15 min, where these rats had a higher glucose peak compared to both FR and SC groups ($p<0.05$). At 60 and 90 min post gavage, the RMS rats had higher glucose concentrations compared to FR rats, although the concentrations were similar to rats in the SC group. Random blood sampling and fasting glucose levels were similar between the RMS rats and those in the FR and SC groups. Taken together, it can be concluded that in the RMS rats, there were perturbations in the glucose disposal following on oral glucose challenge.

The insulin response to an oral glucose challenge did not differ significantly between the rats from the FR and RMS groups. A statistically significant difference was noted at baseline, at which time, the RMS group had higher fasting insulin compared to the other dietary groups ($p<0.05$). However, there was a statistically significant interaction of the effects of treatment*time ($p<0.001$). A graph of this interaction shows higher insulin concentrations in both the RMS and FR groups compared to the SC group. These pieces of data suggest that the AR process, rather than FR ingestion had an effect on insulin secretion in response to a glucose challenge.

With respect to examining whether early introduction of high FR result in changes in body weight, the results from this cohort suggest that early FR-feeding may not lead to increased body weight in later life. The inconsistencies in our findings between this group of animals and those reported in chapter 2 warrant speculation on the potential sources of variability between the two cohorts. First, housing conditions varied slightly between the

cohorts. Second, the changes in body weight and fat mass described in chapter 2 are slight and may be sensitive to alterations in environmental conditions.

The methods and materials used in this experiment were the same as those employed in cohort #1 as described in chapter 2. Food intake was similar between the first group of rats and this group; however, the rats in this cohort had lower body weights than the first group. It is possible that energy was re-routed toward maintenance of other bodily functions, such as core temperature, instead of tissue accretion and growth. Therefore, we speculate that stress, in this case, decreased humidity may have interfered with energy balance. During the period under which the investigations reported in this chapter took place, ambient housing conditions were questionable due to the discontinuation of central humidity regulation. In cohort # 1, the humidity level in the whole animal unit was regulated centrally. However, in subsequent time, the main humidifier was turned off and in its place; a small portable humidifier was installed in the room that housed our rats. Although efforts were made to maintain optimal humidity, there is a possibility that the relative humidity in the room was decreased compared to previous study conditions. One physiological parameter that is affected by high levels of stress is decreased food intake resulting in lower body weight (40,41). There are few reports on the effects of different housing conditions on body weight alterations in the rat. However, stressors such as heat exposure can lead to decreased food intake and subsequently lower body weight (42,43). Harikai et al (44) reported that rats exposed to 37^0C for 60 min daily, for 2 weeks, ate less food and had lower body weight in comparison to the rats kept at 24^0C ($p<0.05$ for both). Corticosterone levels measured immediately after the 60 min of heat exposure was elevated in the stressed rats compared

to those not subjected to the heat stress ($p<0.05$). Retana-Marquez demonstrated that rats exposed to daily doses of stress such as immobilization or immersion in cold water had elevated plasma corticosterone levels compared to control rats ($p<0.05$) (45). The mean body weight gain, measured over a 20 day period, was less in the stressed animals than in controls. For example, control rats had a mean weight gain of 3.35 ± 0.43 g/day, whereas, rats that were subjected to daily bouts of 60 min of immobilization gained an average of 0.86 ± 0.73 g/day ($p<0.05$). Although the types of stress used in these studies are different from an environmental one such as reduced humidity, it is possible that varying humidity affected growth in our rats. Measurement of plasma corticosterone levels may help to clarify whether stress was one factor that interfered with outcome measures in this group of animals.

In summary, this study examined the effects of a short period (7 days) of high FR intake during the suckling to weaning transition on body weight in later life. In this cohort of rats, it did not result in increased body weight. However, metabolic responses to the early dietary modification may be small, and as such, are susceptible to alterations in housing conditions. Because of the sensitive nature of the metabolic changes induced, strict regulation of the environment in future experiments may reduce potential confounding factors, thus allowing these subtle differences to be expressed.

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6. Tables and figures for chapter 3

Table 1. Plasma glucose, insulin, triglyceride and leptin concentrations during the post-weaning period

Parameters	Diet	wk 6	wk 8	wk 11	P value
glucose (mM)	Fr	8.5 ± 0.4	8.4 ± 0.4	8.8 ± 0.4*	*p≤0.05 (FR vs. SC)
	Sc	8.7 ± 0.6	8.7 ± 0.6	7.4 ± 0.6	
	RMS	8.7 ± 0.43	9.0 ± 0.42	8.6 ± 0.40	
insulin (ng/mL)	Fr	0.51 ± 0.06	0.61 ± 0.07	0.77 ± 0.10	
	Sc	0.57 ± 0.07	0.54 ± 0.09	0.73 ± 0.13	
	RMS	0.48 ± 0.07	0.72 ± 0.08	1.04 ± 0.11	
triglyceride (mg/dL)	Fr	107 ± 6	147 ± 13	141 ± 17	
	Sc	115 ± 8	122 ± 15	119 ± 21	
	RMS	109 ± 6	126 ± 13	151 ± 17	
leptin (ng/mL)	Fr	3.9 ± 0.5	8.2 ± 0.8	11.1 ± 1.5	
	Sc	4.1 ± 0.6	7.2 ± 1.0	12.2 ± 1.6	
	RMS	3.1 ± 0.5	9.4 ± 0.8	12.5 ± 1.6	

Data are mean ± SEM

Table 2. Fasted plasma glucose, insulin and triglyceride levels at 10 weeks of age

Parameters	Diet	wk 10	P value
glucose (mM)	Fr	6.3 ± 0.3	
	Sc	6.2 ± 0.4	
	RMS	7.1 ± 0.4	
insulin (ng/mL)	Fr	0.13 ± 0.04	* p≤0.05 (RMS vs. FR & SC)
	Sc	0.11 ± 0.06	
	RMS	0.26 ± 0.04*	
triglyceride (mg/dL)	Fr	92 ± 8	
	Sc	82 ± 10	
	RMS	102 ± 8	

Data are mean ± SEM

Table 3. Liver and epidydimal fat weight of animals in the three dietary treatment groups

Diet	Liver	Epidydimal fat pad
	absolute weight (g) *(g/100g bodyweight)*	
FR (n =15)	22.7 ± 1.0 *4.3 ± 0.1*	5.1 ± 0.6 *1.0 ± 0.1*
RMS (n =15)	24.4 ± 1.0* *4.5 ± 0.1*†	6.2 ± 0.6 *1.0 ± 0.1*
SC (n =10)	20.9 ± 1.3 *4.0 ± 0.2*	5.0 ± 0.7 *1.0 ± 0.1*

Data are mean ± SEM. * $p \leq 0.05$ RMS vs. SC, † $p \leq 0.05$ RMS vs. SC

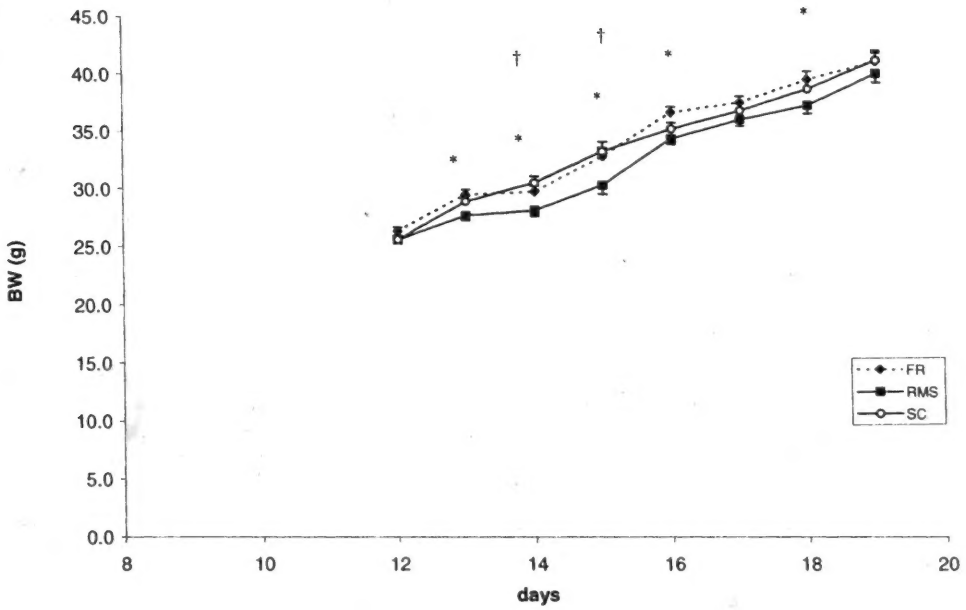

Fig. 1A. Body weight of pups during the artificial rearing (FR & RMS groups) or suckling period (SC group) from days 12 to 19 postnatal; expressed as mean ± SEM; * $p<0.05$ FR vs. RMS, $^{\dagger}p<0.05$ SC vs RMS.

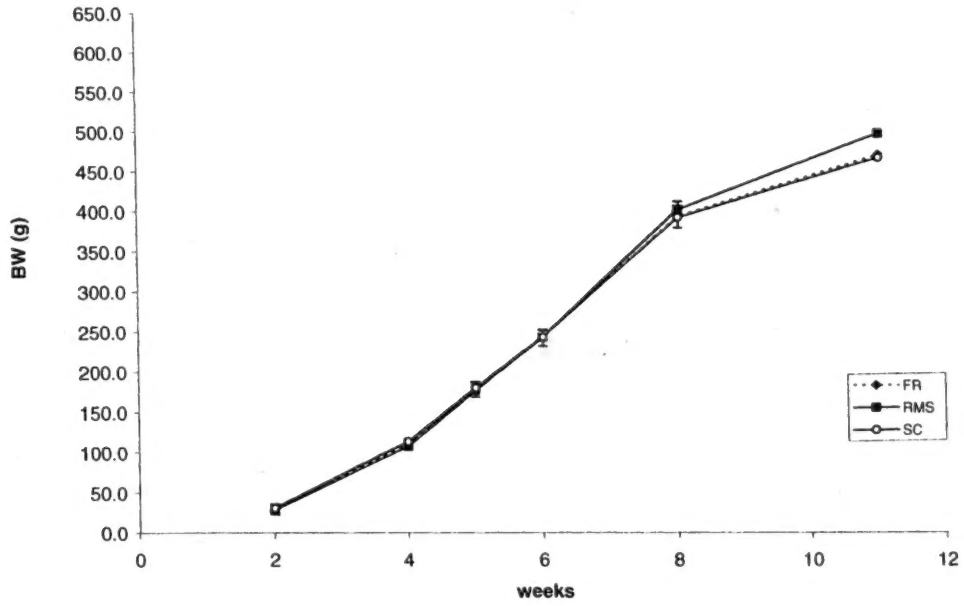

Fig. 1B. Body weight of rats in the three dietary treatment groups from 2 to 11 weeks of age. At 2 weeks of age, the pups were either suckling on the dam or artificially reared. All pups were weaned at 19 days of age onto a purified rat chow and were maintained on this diet until they were 11 weeks of age; expressed as mean ± SEM.

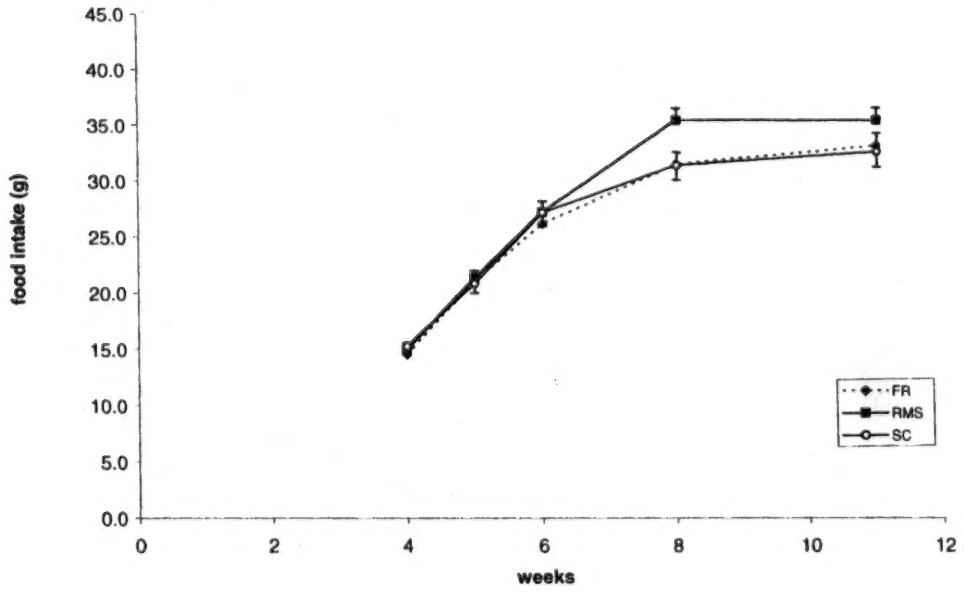

Fig. 2. Food intake of rats in the three dietary groups from weeks 4 to 11; expressed as mean ± SEM.

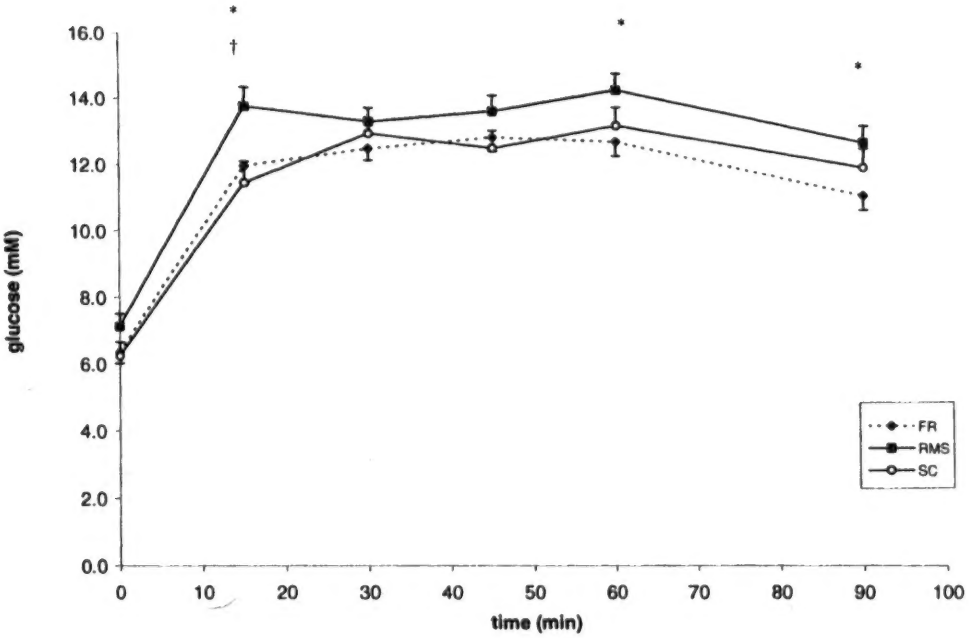

Fig. 3A. Changes in plasma glucose levels following a glucose challenge of 3g/kg BW in the three dietary treatment groups; expressed as mean ± SEM; $^{*}p<0.05$ RMS vs. FR, $^{\dagger}p<0.05$ RMS vs. SC.

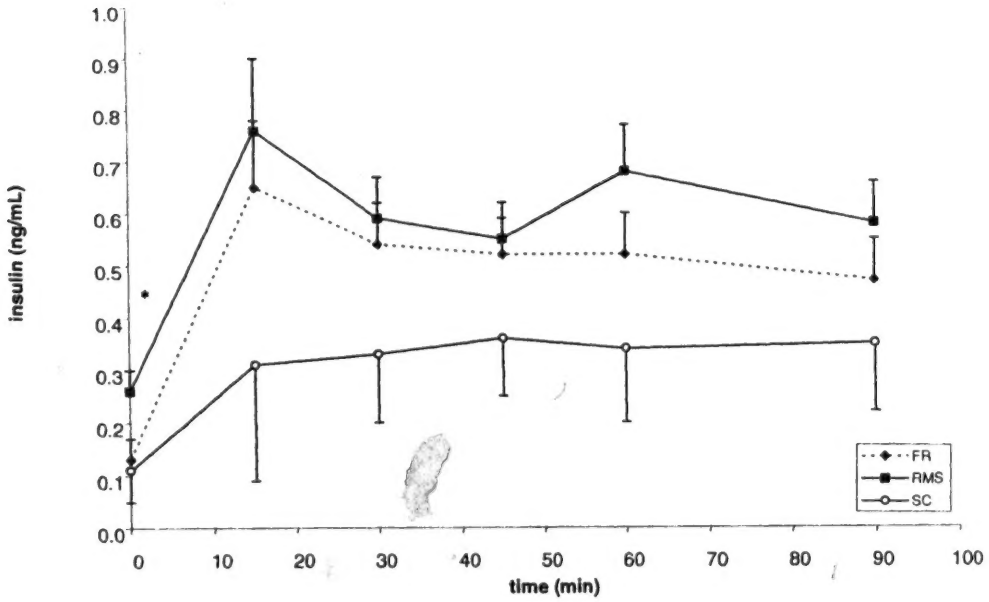

Fig. 3B. Plasma insulin levels in response to a glucose challenge of 3g/kg BW in the three dietary treatment groups; expressed as mean ± SEM; * $p<0.05$ RMS vs. FR.

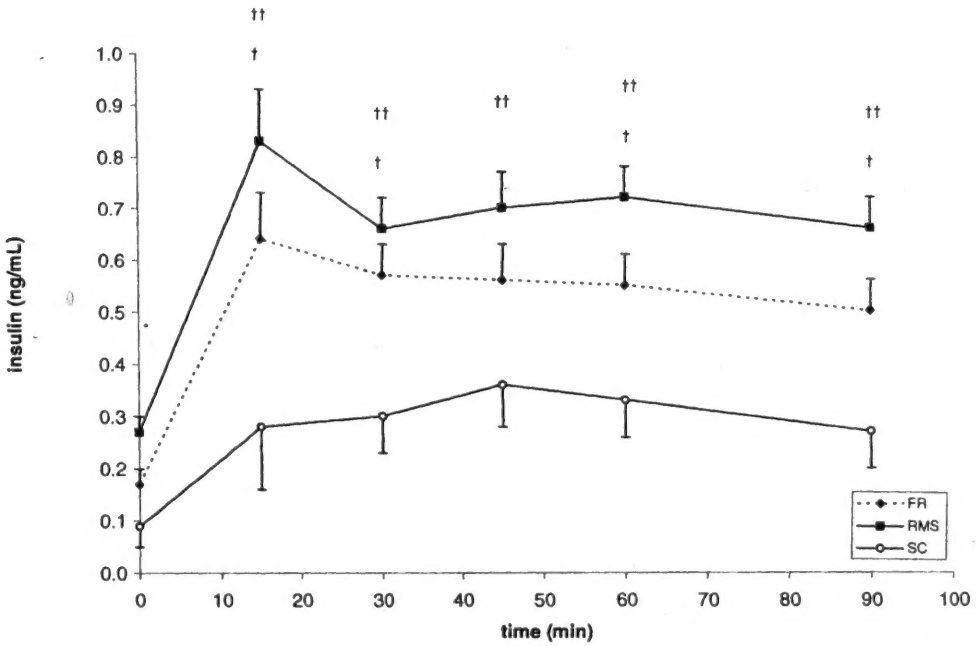

Fig. 3C. Graph of the treatment*time interaction for the plasma insulin concentrations observed during the oral glucose tolerance test; expressed as mean ± SEM; * $p< 0.05$ RMS vs. FR & SC, † $p< 0.05$ FR vs. SC, †† $p< 0.001$ RMS vs. SC.

Chapter 4: Summary and future directions

1. Summary of results

My project attempted to bridge the gap between two bodies of knowledge – metabolic programming and fructose metabolism. Currently, the literature on programming suggests that the suckling period is one phase of growth during which dietary modifications can alter metabolism. Although the evidence on the duration of impact varies between studies, it seems that the effects on body composition and growth may persist into adulthood. Similarly, the evidence on the effects of FR intake and body weight is inconclusive; however, chronic FR ingestion can lead to altered substrate utilization. Based on this information, we hypothesized that introduction of FR during the suckling period can alter metabolism such that weight gain is promoted in later life.

Results reported in chapter 2 collectively demonstrated that a short period of high FR intake during the suckling phase of the lifespan changed metabolism such that body weight is increased in adulthood. Specifically, the early FR-fed rats had increased long chain fatty acid uptake into skeletal muscles, higher circulating insulin and leptin concentrations in later life. In the subsequent investigation, the results of which are presented in chapter 3, artificial rearing affected insulin sensitivity as suggested by increased insulin response to a glucose challenge. Fructose feeding did not have an independent effect on glucose metabolism or body weight in adulthood. This may be due to potential confounding factors that were beyond our control.

Although this set of studies did not conclusively answer our hypothesis, it generated other interesting findings. The main one is that the alterations resulting from 7

days of FR intake during suckling in rats is small and as such, any changes incurred may be sensitive to alterations in stress induced by different housing conditions.

2. Future Directions

2.1. At the level of the whole body

With the exception of fatty acid uptake, the majority of our outcome measures looked at changes in metabolism on the whole body level. Two additional outcome measures that might add to our understanding of the potential metabolic programming effects of early FR introduction are: measuring energy expenditure and extending or re-introducing the high FR feeding regime.

a) In our experiment, we attempted to understand the intake and metabolism side of the energy homeostasis equation. Another outcome measure that can be examined is energy expenditure. Some investigators have shown that activity levels may be programmed during early development (1). By determining the activity level in future studies, we may be able to understand both sides of the energy balance equation.

b) In the context of programming, introducing a secondary insult in the form of a highly palatable diet, such as the cafeteria diet, or re-introduction of the high FR feeding regime may amplify the effects of early FR ingestion. This could be done immediately following the weaning transition or at a later phase of growth in the postnatal period.

2.2. At the cellular level

Body weight represents the cumulative effects of a concert of metabolic functions. In order to appreciate the underlying effects of high FR feeding, changes at the cellular level such as the rate of fatty acid utilization and protein tyrosine phosphatase 1B activity should be measured. This information would help to shed light onto the possible pathways that can be impacted by FR metabolism.

a) Fatty acid accumulation may interfere with the process of insulin signaling (2). Data presented in chapter 2 illustrated that palmitate uptake into muscles of FR-fed rats were elevated. Determination of the rate of fatty acid metabolism (both, esterification and oxidation) in skeletal muscle may help to separate out the differential between these two processes, thus enabling us to determine whether there is accumulation of fatty acid molecules in the muscle.

b) Protein tyrosine phosphatase 1B (PTP1B) is one biological regulator of the insulin signaling cascade and up-regulation of this molecule attenuates insulin signaling (3). It is possible that insulin resistance observed in FR-fed animals may be a result of increased PTP1B activity (4). Our results in chapter 2 showed that early FR-feeding resulted in increased circulating insulin levels. Examining the level and activity of this enzyme may help to elucidate the behaviour of insulin signaling in skeletal muscles.

2.3. Focus on stress

We speculated that varying levels of humidity may been a differential stressor on the rats in cohorts 2 and 3, reported in chapter 3. Consequently, these animals may have re-routed energy expenditure toward the maintenance of bodily functions such as the control of core body temperature. Potential parameters that can help to clarify this issue are measurements of body temperature, plasma corticosterone concentration, and uncoupling protein 1 in brown adipose tissue.

a) Having a record of body temperatures over the study period, measured via a rectal thermometer, is useful in establishing body temperature over the study period. Deviations from normal ranges suggest alterations in energy utilization.

b) Determination of corticosterone concentrations will help to quantify the level of stress experienced by the rats.

c) Uncoupling protein 1 is expressed in brown adipocytes. Its main function is to generate heat by uncoupling substrate oxidation from adenosine-5'-tripophosphate synthesis in the respiratory chain, and in chronic cold conditions, UCP1 expression is increased (5). Measurement of UCP1 expression and concentration in brown adipose tissue will provide information on the thermogenesis activity, thus offering a clue on energy regulation.

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